FORM-PTO-1390 (Rev. 12-29-99) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 031309-003

U.S. APPLICATION NO. (If known, see 37 C F R 1 5): 09/647543

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/02243	1 April 1999	2 April 1998

TITLE OF INVENTION: PROMOTER AND CONSTRUCTIONS FOR EXPRESSION OF RECOMBINANT PROTEINS IN FILAMENTOUS FUNGI

APPLICANT(S) FOR DO/EO/US: Heidi Sisniega BARROSO et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Sequence Listing in computer readable form **and** a paper copy.

528 Rec'd PCT/PTO 02 OCT 2000

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
Unassigned 09/647543	PCT/EP99/02243	031309-003

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000.00 (960)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00 (970)		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00 (958)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00 (956)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00 (962)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00 (154)** for furnishing the oath or declaration later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	61 -20 =	41	X$18.00 (966)	$ 738.00	
Independent Claims	9 -3 =	6	X$80.00 (964)	$ 480.00	
Multiple dependent claim(s) (if applicable)			+$270.00 (968)	$	
TOTAL OF ABOVE CALCULATIONS =				$ 2,078.00	
Reduction for 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	-
SUBTOTAL =				$ 2,078.00	
Processing fee of **$130.00 (156)** for furnishing the English translation later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$ 2,078.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 (581)** per property +				$	
TOTAL FEES ENCLOSED =				$ 2,078.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 2,078.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 02-4800 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

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NAME

32,858
REGISTRATION NUMBER

Patent
Attorney's Docket No. 031309-003

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Heidi Sisniega BARROSO et al.	)	Group Art Unit: Unassigned
	)	
Application No.: Unassigned	)	Examiner: Unassigned
	)	
Filed:	)	
	)	
For: PROMOTER AND CONSTRUCTIONS FOR EXPRESSION OF RECOMBINANT PROTEINS IN FILAMENTOUS FUNGI	)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to an examination on the merits, please amend the above identified application as follows:

IN THE SPECIFICATION:

On substitute sheet 2A, line 1, delete "Gene (1983) ," and insert therefor --Gene (1983),--;

line 2, delete "Neurospora crassa" and insert therefor --Neurospora crassa--;

line 3, delete "Appl. Microbiol. Biotechnol. (1997), Vol." and insert therefor --Appl. Microbiol. Biotechnol. (1997), vol.--

On page 3, lines 4 and 9, delete both instances of "SEQ ID No. 1" and insert therefor --SEQ ID NO:1--.

On page 3, line 7, change "Aspergillus nidulans" to --Aspergilus nidulans--

On page 3, line 18, delete "SEQ ID No. 1" and insert therefor --SEQ ID NO:1--;

On page 4, lines 1, 4, 12 and 18, delete all instances of "SEQ ID No. 1" and insert therefor --SEQ ID NO:1--;

line 33, delete "SEQ ID No. 2" and insert therefor --SEQ ID NO:2--.

On page 4, line 15, change "Aspergillus nidulans" to --Aspergilus nidulans--

On page 6, line 27, delete "SEQ ID No. 1" and insert therefor --SEQ ID NO:1--.

On page 8, line 7, delete "SEQ ID No. 1" and insert therefor --SEQ ID NO:1--.

On page 12, line 13, delete "Genebank" and insert therefor --Genbank--.

On page 20, lines 19 and 22, delete both instances of "SEQ ID No. 1" and insert therefor --SEQ ID NO:1--.

On page 21, line 14, delete "SEQ ID No. 2" and insert therefor --SEQ ID NO:2--.

On page 22, line 1, delete "A.nidulans" and insert therefor --A. nidulans--.

On page 24, line 14, delete "SEQ ID No. 1" and insert therefor --SEQ ID NO:1--.

On page 28, line 22, delete "paralell" and insert therefor –parallel–.

On page 35, line 16, delete "Cat.No." and insert therefor -Cat. No.-.

IN THE CLAIMS:

Please note that the claims on amended pages 1-5 attached to the International Preliminary Examination Report (Annexes) and submitted herewith, have replaced the originally filed pages 48-52 of the application. The claims to be examined and amended by this preliminary amendment are found on amended pages 1-5.

Please cancel claims 16, 17 and 35 without prejudice or disclaimer as to the subject matter contained therein.

Please amend the claims of amended pages 1-5 as follows:

1. (Amended) An isolated nucleic acid comprising a [A] promoter for the expression of recombinant proteins in filamentous fungi that comprises a nucleotide sequence [-] or a complementary strand thereof [-] selected from the group consisting of: (a) nucleotides [the nucleotide sequence numbered] 1-740 of [in the enclosed SEQ ID No. 1] SEQ ID NO:1; and (b) a nucleotide sequence that hybridizes under stringent conditions to that defined in (a), with the proviso, that the nucleotide sequence is not the promoter of the [gdh] *gdh* gene [for] from *Aspergillus nidulans* [Aspergillus nidulans].

2. (Amended) An isolated nucleic acid [A promoter] according to claim 1, wherein the promoter [which has] consists of [the sequence of] nucleotides [numbered] 1-740 [in SEQ ID No. 1] of SEQ ID NO:1 or its complementary strand.

3. (Amended) An isolated nucleic acid comprising a [Isolated] promoter of a glutamate dehydrogenase gene from a fungus of the genus *Aspergillus* [Aspergillus] with

the proviso[,] that the sequence is not the promoter of the *gdh* [gdh] gene from *Aspergillus nidulans* [Aspergillus nidulans].

4. (Amended) The isolated nucleic acid [Isolated promoter] according to claim 3, wherein the fungus is *Aspergillus awamori* or *Aspergillus niger* [Aspergillus awamori or Aspergillus niger].

5. (Amended) The isolated nucleic acid [Isolated promoter] according to claim 4, wherein the fungus is *Aspergillus awamori* [Aspergillus awamori].

6. (Amended) A purified and isolated DNA sequence that encodes a glutamate dehydrogenase protein and that comprises a nucleotide sequence [-] or a complementary strand thereof [-] selected from the group consisting of: (a) nucleotides [the nucleotide sequence numbered] 741-2245 [in the enclosed SEQ ID No. 1] of SEQ ID NO:1; and (b) a nucleotide sequence that hybridizes under stringent conditions to that defined in (a), with the proviso[,] that the sequence is not the [gdh] *gdh* gene from *Aspergillus nidulans* [Aspergillus nidulans].

7. (Amended) [A] The DNA sequence according to claim 6, [which has the sequence of] consisting of nucleotides [numbered 741-2242 in SEQ ID No. 1] 741-2245 of SEQ ID NO:1, or its complementary strand.

8. (Amended) An isolated DNA sequence encoding a glutamate dehydrogenase from a fungus of the genus *Aspergillus*, [Aspergillus,] with the proviso[,] that the sequence is not the [gdh] *gdh* gene from *Aspergillus nidulans* [Aspergillus nidulans].

9. (Amended) The [An] isolated DNA sequence according to claim 8, wherein the fungus is *Aspergillus awamori* or *Aspergillus niger* [Aspergillus awamori or Aspergillus niger].

10. (Amended) The [An] isolated DNA sequence according to claim 9, wherein the fungus is *Aspergillus awamori* [Aspergillus awamori].

11. (Amended) A [The] protein encoded by any of the DNA sequences according to claim 6.

12. (Amended) A [The] protein [which has the amino acid sequence is SEQ ID No. 2] comprising SEQ ID NO:2.

13. (Amended) An isolated glutamate dehydrogenase from a fungus of the genus *Aspergillus* [Aspergillus] with the proviso[,] that the glutamate dehydrogenase is not the glutamate dehydrogenase from *Aspergillus nidulans* [Aspergillus nidulans].

14. (Amended) The [An] isolated glutamate dehydrogenase according to claim 13, wherein the fungus is *Aspergillus awamori* or *Aspergillus niger* [Aspergillus awamori or Aspergillus niger].

15. (Amended) The [An] isolated glutamate dehydrogenase according to claim 14, wherein the fungus is *Aspergillus awamori* [Aspergillus awamori].

18. (Amended) A DNA [construction] construct [that comprises] comprising: a) a promoter from a glutamate dehydrogenase gene from a fungus of the genus *Aspergillus* [Aspergillus]; b) a DNA sequence encoding a protein [normally] expressed from a filamentous fungus or a portion thereof; c) a DNA sequence encoding a cleavable linker peptide; and d) a DNA sequence encoding a desired protein.

19. (Amended) A DNA [construction] construct that comprises: a) a promoter from a glutamate dehydrogenase gene from a fungus of the genus *Aspergillus*; b) a DNA sequence encoding a protein expressed from a filamentous fungus or a portion thereof; c) a

DNA sequence encoding a cleavable linker peptide; and d) a DNA sequence encoding a desired protein [according to claim 18], wherein the promoter under a) is a promoter according to claim 1 [any one of claims 1 to 5].

20. (Amended) The [A] DNA [construction] construct according to claim 18, wherein the DNA sequence under b) encodes a protein or portion thereof selected from the group consisting of: i) glucoamylase from *Aspergillus awamori, Aspergillus niger, Aspergillus oryzae* or *Aspergillus sojae* [Aspergillus awamori, Aspergillus niger, Aspergillus oryzae or Aspergillus sojae]; ii) B2 from *Acremonium chrysogenum* [Acremonium chrysogenum]; and iii) a glutamate dehydrogenase from a filamentous fungus.

21. (Amended) The [A] DNA [construction] construct according to claim 20, wherein the DNA sequence under b) encodes a glucoamylase from *Aspergillus awamori, Aspergillus niger, Aspergillus oryzae* or *Aspergillus sojae* [Aspergillus awamori, Aspergillus niger, Aspergillus oryzae or Aspergillus sojae] or a portion thereof.

22. (Amended) The [A] DNA [construction] construct according to claim 20, wherein the DNA sequence under b) encodes [the] protein B2 from *Acremonium chrysogenum* [Acremonium chrysogenum] or a portion thereof.

23. (Amended) The [A] DNA [construction] construct according to claim 20, wherein the DNA sequence under b) encodes a glutamate dehydrogenase from a filamentous fungus or a portion thereof.

24. (Amended) The [A] DNA [construction] construct according to claim 18, wherein the DNA sequence under c) contains a KEX2 processing sequence.

25. (Amended) The [A] DNA [construction] construct according to claim 18 [any one of claims 18 to 24], wherein the DNA sequence under d) encodes thaumatin.

26. (Amended) The [A] DNA [construction] construct according to claim 25, wherein the DNA sequence under d) is the thaumatin II synthetic gene from plasmid pThIX [disclosed in EP 684312].

27. (Amended) A DNA [construction] construct comprising a gdh promoter from a fungus of the genus *Aspergillus* [Aspergillus] operatively linked to a DNA sequence encoding a [recombinant] desired protein.

28. (Amended) A DNA [construction] construct comprising a gdh promoter from a fungus of the genus *Aspergillus* operatively linked to a DNA sequence encoding a desired protein [according to claim 27], wherein the promoter is a promoter according to claim 1 [any one of claims 1 to 5].

29. (Amended) A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct [construction] according to claim 18 [any one of claims 18 to 28].

30. (Amended) The [A] culture according to claim 29, wherein the filamentous fungus is a fungus from the genus *Aspergillus* [Aspergillus].

31. (Amended) The [A] culture according to claim 29, wherein the filamentous fungus is selected from the group consisting of *Aspergillus awamori, Aspergillus niger, Aspergillus oryzae, Aspergillyus nidulans* and *Aspergillus sojae* [Aspergillus awamori, Aspergillus niger, Aspergillus oryzae, Aspergillyus nidulans and Aspergillus sojae].

32. (Amended) A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid [according to claim 29], wherein the plasmid contains a DNA [construction] construct according to claim 25 [any one of claims 25 or 26].

33. (Amended) A process for producing a recombinant protein in a filamentous fungus comprising [the following steps]:

a) preparing [preparation of] an expression plasmid containing a DNA construct according to claim 18 [any of claims 18 to 28];

b) transforming [transformation of] a strain of filamentous fungus with said expression plasmid;

c) culturing [culture of] the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying [depending on the case, separation and purification of] the desired protein from the fermentation broth to produce the recombinant protein.

34. (Amended) The [A] process according to claim 33, wherein the recombinant protein is thaumatin and the expression plasmid contains a DNA construct [construction] according to claim 25 [claims 25 or 26].

Please add the following new claims:

--36. A method for expressing a recombinant protein in filamentous fungi comprising:

(a) preparing a nucleic acid comprising a promoter from a glutamate dehydrogenase gene from a fungus of the genus *Aspergillus* operably linked to a second nucleic acid encoding a protein;

(b) inserting said nucleic acid into a filamentous fungi; and

(c) culturing the filamentous fungi to express the recombinant protein.

37. The method of Claim 36, wherein the promoter is selected from the group consisting of:

(a) nucleotides 1-740 of SEQ ID NO:1;

(b) a nucleotide sequence that hybridizes under stringent conditions to nucleotides 1-740 of SEQ ID NO:1;

(c) a promoter of a glutamate dehydrogenase gene from a fungus of the genus *Aspergillus* with the proviso that the sequence is not the promoter of the *gdh* gene from *Aspergillus nidulans*; and

(d) a promoter of a glutamate dehydrogenase gene of *Aspergillus awamori* or *Aspergillus niger*.

38. A method of isolating a glutamate dehydrogenase gene comprising hybridizing a nucleotide sequence according to claim 1 to a nucleic acid under stringent conditions.

39. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 19.

40. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 20.

41. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 21.

42. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 22.

43. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 23.

44. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 24.

45. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 25.

46. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 26.

47. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 27.

48. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 28.

49. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 19;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

50. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 20;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

51. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 21;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

52. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 22;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

53. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 23;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

54. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 24;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

55. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 25;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

56. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 26;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

57. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 27;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

58. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 28;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

59. A DNA construct that comprises: a) a promoter from a glutamate dehydrogenase gene from a fungus of the genus *Aspergillus*; b) a DNA sequence encoding a protein expressed from a filamentous fungus or a portion thereof; c) a DNA sequence encoding a cleavable linker peptide; and d) a DNA sequence encoding a desired protein, wherein the promoter under a) is a promoter according to claim 3.

60. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 59.

61. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 59;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.

62. A DNA construct comprising a gdh promoter from a fungus of the genus *Aspergillus* operatively linked to a DNA sequence encoding a desired protein, wherein the promoter is a promoter according to claim 3.

63. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construct according to claim 62.

64. A process for producing a recombinant protein in a filamentous fungus comprising:

a) preparing an expression plasmid containing a DNA construct according to claim 62;

b) transforming a strain of filamentous fungus with said expression plasmid;

c) culturing the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and

d) separating and purifying the desired protein from the fermentation broth to produce the recombinant protein.--

REMARKS

The Examiner's attention is drawn to the amendments to the application made in the Preliminary Examination Report, and for the convenience of the Examiner the following items are submitted with this application:

A) International PCT Publication
B) Preliminary Examination Report
C) Substitute Sheets

The amendments to the specification corrects typographical errors and does not introduce any prohibited new matter.

Claims 1-15 and 18-34 have been amended to more distinctly claim the subject matter of the invention and place the claims in proper form in accordance with U.S. Patent Office procedures.

New claims 36-38 replace claims 16-17 and 35 respectively. Support for the new claims can be found, at least, in the claims as originally filed. No prohibited new matter is introduced by the amendment to the claims or by entry of the new claims.

New claims 39-48 replace the multiple dependencies of claim 29, and new claims 49-58 replace the multiple dependencies of claim 33. New claims 59-61 replace the multiple dependencies of claims 19, 29 and 33, respectively. New claims 62-64 replace the multiple dependencies of claims 28, 29 and 33, respectively.

Also, Applicants have amended the application to substitute the originally filed claim pages 48-52 with the amended claim pages 1-5 attached to the International Preliminary Examiner Report (Annexes) and included in the application as filed herewith.

Applicants retain the right to reintroduce any subject matter canceled by the present Amendment at any time during the prosecution of this application or any continuation or divisional thereof in the United States. It is believed that no prohibited new matter is being introduced by entry of this paper.

In view of the foregoing, an action on the merits is now believed to be in order. Such action is earnestly solicited.

In the event that there are any questions relating to this application, it would be appreciated if the Examiner would telephone the undersigned attorney concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: Reg. No. 32,958
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Date: October 2, 2000

Promoter and constructions for expression of recombinant proteins in filamentous fungi

This invention relates to improvements in the expression of proteins, particularly of fusion proteins, by recombinant DNA technology, using filamentous fungi as the host. These improvements refer mainly to the use of a new promoter and new DNA constructions containing it.

DESCRIPTION OF THE PRIOR ART

Filamentous fungi are known to produce in nature a wide range of homologous proteins in large amounts. For this reason, filamentous fungi have been regarded as attractive hosts for the expression of recombinant proteins. For instance, Aspergillus awamori has been used for the production of recombinant proteins such as bovine chymosin and human lactoferrin.

Some recombinant proteins, however, have proved to be very difficult to express in filamentous fungi. This is the case for example of interleukin-6 and thaumatin. The thaumatins are proteins with a very sweet taste and the ability to increase the palatability of food. In industry they are currently extracted from the arils of the fruit of the plant Thaumatoccocus daniellii Benth (M.Witty, J.D. Higginbotham, Thaumatin ,1994, CRC Press, Boca Ratón, Florida). Thaumatins can be isolated from these arils in at least five different forms (I, II, III, b and c), thaumatins I and II being the most abundant types in the arils. Despite its advantages, industrial use of thaumatins of plant origin is very limited because of the extreme difficulty involved in obtaining the fruit from which it is extracted. Attempts have been made to produce thaumatins by genetic engineering in different hosts such as bacteria, yeasts and transgenic plants, but until now the results have been considered disheartening and thus the thaumatin available to industry is very scarce and expensive.

European patent EP 684312 describes a process for preparing recombinant thaumatin in filamentous fungi. One problem of this process is that the yields obtained are low in comparison with those needed for industrial production of thaumatins.

It is known in the art that yields of recombinant proteins can be improved when the recombinant protein of interest is expressed as a fusion with another protein, and when expression of this cassette is driven by a strong fungal promoter. This other protein, named "carrier protein", is usually a highly expressed protein of fungal origin. Up to now, the most frequently used expression system involves the glucoamylase promoter and gene from Aspergillus awamori as the promoter and the carrier protein, respectively (P.P. Ward et al., Biotechnology 1995, vol. 13, pp. 498-502). However, in some cases the use of this expression system does not lead to high levels of the desired recombinant protein. One of these specially problematic cases is the expression of recombinant thaumatin in filamentous fungi.

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In view of the above, it is clear that there is the need to provide new and more efficient expression systems that allow the production of higher concentrations of those proteins that are difficult to express in filamentous fungi, such as thaumatins. This goal is achieved with the new promoter and DNA constructions provided in the present invention, as explained below.

DESCRIPTION OF THE INVENTION

The present invention provides a new expression system that makes use of the promoter from the glutamate dehydrogenase (gdh) gene from filamentous fungi of the genus Aspergillus, particularly from Aspergillus awamori.

One of the objects of the present invention is a new promoter for the expression of recombinant proteins in

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Gene (1983) , vol. 26, pp. 253-260 discloses the complete nucleotide sequence of the Neurospora crassa NADP-specific glutamate dehydrogenase gene.

Appl. Microbiol. Biotechnol. (1997), Vol. 47, pp 1-11 discloses the efficient production of secreted proteins by Aspergillus. Particular focus is laid on the gene fusion strategies.

EP 0 684 312 A2 relates to a preparation process of a natural protein sweetener, thaumatin. Said document discloses a new nucleotide sequence encoding thaumatin with optimised codon usage for expression in filamentous fungi.

filamentous fungi that comprises a nucleotide sequence - or a complementary strand thereof - selected from the group consisting of: (a) the nucleotide sequence numbered 1-740 in the enclosed SEQ ID No. 1; and (b) a nucleotide sequence that hybridizes under stringent conditions to that defined in (a) with the proviso that the sequence is not the promoter of the gdh gene from Aspergillus nidulans. Particularly preferred is the promoter comprising the sequence defined in (a), i.e. the nucleotide sequence numbered as 1-740 in SEQ ID No. 1, which corresponds to the gdhA promoter of the glutamate dehydrogenase A gene from Aspergillus awamori.

Although glutamate dehydrogenase A disclosed herein is the first glutamate dehydrogenase identified and described in the filamentous fungus Aspergillus awamori, there may exist other glutamate dehydrogenases in Aspergillus awamori. The novel nucleotide sequence of the Aspergillus awamori gdhA promoter and/or gene shown in SEQ ID No. 1 or a portion thereof can be used as a probe for the identification and isolation of other homologous promoters/genes of glutamate dehydrogenases in Aspergillus awamori as well as in other organisms, preferably in filamentous fungi, more preferably in fungi of the genus Aspergillus, still more preferably in Aspergillus awamori and Aspergillus niger, and specially in Aspergillus awamori, following the teachings of the present invention. Consequently, the present invention is not limited to the specific gdhA promoter from Aspergillus awamori disclosed herein but also relates to the promoter of any glutamate dehydrogenase gene from a fungus of the genus Aspergillus with the proviso that it is not from Aspergillus nidulans. Examples of said Aspergilli include Aspergillus awamori, Aspergillus niger, Aspergillus oryzae and Aspergillus sojae. In a preferred embodiment, the invention relates to a promoter of a glutamate dehydrogenase gene from Aspergillus awamori or Aspergillus niger. In a more preferred embodiment, the invention relates to a promoter of a glutamate dehydrogenase gene from Aspergillus awamori. The

use of the novel nucleotide sequence shown in SEQ ID No. 1 or a portion thereof as probe is also a object of the present invention. The term "a portion thereof" denotes any part of the nucleotide sequence of SEQ ID No.1 that is functional as a probe.

Another object of the present invention is a new DNA sequence, purified and isolated, that encodes a glutamate dehydrogenase protein and that comprises a nucleotide sequence - or a complementary strand thereof - selected from the group consisting of: (a) the nucleotide sequence numbered 741-2245 in the enclosed SEQ ID No. 1; and (b) a nucleotide sequence that hybridizes under stringent conditions to that defined in (a) with the proviso that the sequence is not the gdh gene from Aspergillus nidulans. In a preferred embodiment, the nucleotide sequence encoding a glutamate dehydrogenase is the sequence defined in (a), i.e. the nucleotide sequence numbered as 741-2245 in SEQ ID No. 1. The present invention is not limited, however, to the specific gdhA gene from Aspergillus awamori disclosed herein but also relates to any glutamate dehydrogenase gene from a fungus of the genus Aspergillus with the proviso that it is not from Aspergillus nidulans. In a preferred embodiment, the invention relates to the DNA sequences encoding glutamate dehydrogenase from Aspergillus awamori or Aspergillus niger. In a more preferred embodiment, the invention relates to the DNA sequences encoding glutamate dehydrogenase from Aspergillus awamori.

Another object of the invention are the novel proteins encoded by any of the DNA sequences defined above. In a preferred embodiment, this protein has the amino acid sequence shown in the enclosed SEQ ID No. 2. But are also included in the present invention any glutamate dehydrogenase from a fungus of the genus Aspergillus with the proviso that it is not from Aspergillus nidulans, more preferably a glutamate dehydrogenase from Aspergillus awamori or Aspergillus niger, and still more preferably a glutamate dehydrogenase from Aspergillus awamori.

The invention further relates to the use of the glutamate dehydrogenase promoters above described for the expression of recombinant proteins in filamentous fungi. Certain glutamate dehydrogenases from several microorganisms are already known and their genes have been disclosed, in particular the glutamate dehydrogenase A (gdhA) gene from Aspergillus nidulans (A.R. Hawkins et al., Mol. Gen. Genet. 1989, 218(1), pp. 105-111). However, to the best of our knowledge, there has been no disclosure up to now of the expression of a recombinant protein making use of the gdhA promoter from A. nidulans nor has it ever been mentioned that it might be useful for improving the expression of recombinant proteins in filamentous fungi. As shown in the examples below, the glutamate dehydrogenase promoter from Aspergillus awamori has proven to be very strong in promoting transcription of heterologous genes. Therefore, this promoter as well as related ghd promoters from Aspergilli are expected to drive high-level transcription of genes and thus are expected to be of use in the expression of recombinant proteins in filamentous fungi. It is thus a further object of the present invention the use of a promoter from a glutamate dehydrogenase gene from a fungus of the genus Aspergillus for the expression of recombinant proteins in filamentous fungi. Preferably, the gdh promoter is from a fungus of the genus Aspergillus with the proviso that it is not from Aspergillus nidulans, more preferably it is from Aspergillus awamori or Aspergillus niger, still more preferably it is from Aspergillus awamori, and particularly preferably it is one of the novel gdh promoters described above.

There is in principle no limitation on the desired recombinant protein to be expressed. Examples of such desired proteins (which term, as used herein, includes proteins and smaller polypeptides) include, but are not limited to, enzymes, hormones, cytokines, growth factors,

structural proteins, plasma proteins and others. A non-limiting list of examples of proteins that can be expressed includes human proteins such as interferons, interleukins, tissue plasminogen activator, serum albumin, growth hormone, and growth factors. Other proteins can be of non-human origin such as lipases of both fungal and non-fungal origin, proteases, thaumatins, bovine chymosin, etc. Polypeptides, which can be of human and non-human origin, include calcitonin, glucagon, insulin, nerve growth factor, epidermal growth factor, the anticoagulant Hirudin and analogs such as R3-hirulog.

A further object of the present invention are the DNA constructions that comprise: a) a promoter from a glutamate dehydrogenase gene from a fungus of the genus Aspergillus; b) a DNA sequence encoding a protein normally expressed from a filamentous fungus or a portion thereof; c) a DNA sequence encoding a cleavable linker peptide; and d) a DNA sequence encoding a desired protein. In a preferred embodiment, the promoter under a) comprises a gdh promoter from a fungus of the genus Aspergillus with the proviso that it is not from Aspergillus nidulans, more preferably it is from Aspergillus awamori or Aspergillus niger, still more preferably it is from Aspergillus awamori, yet more preferably it comprises any of the new promoters described above, and more particularly it comprises the nucleotide sequence 1-740 in SEQ ID No. 1. The DNA sequence under b) encodes a protein normally expressed from a filamentous fungus or a portion thereof that is functional, i.e. that is capable of producing increased secretion of the desired protein. Examples of such protein under b) include glucoamylase, α-amylase and aspartyl proteases from Aspergillus awamori, Aspergillus niger, Aspergillus oryzae and Aspergillus sojae, cellobiohydrolase I, cellobiohydrolase II, endoglucanase I and endoglucanase III from Trichoderma species, glucoamylase from Neurospora and Humicola species, the protein B2 from Acremonium chrysogenum and a glutamate dehydrogenase from a

filamentous fungi. In a preferred embodiment, the DNA sequence under b) encodes a protein or portion thereof selected from the group consisting of: i) glucoamylase from Aspergillus awamori, Aspergillus niger, Aspergillus oryzae or Aspergillus sojae; ii) B2 from Acremonium chrysogenum; and iii) a glutamate dehydrogenase from a filamentous fungi; more preferably, the DNA sequence under b) encodes a protein or portion thereof selected from the group consisting of: i) glucoamylase from Aspergillus awamori, Aspergillus niger, Aspergillus oryzae or Aspergillus sojae; ii) B2 from Acremonium chrysogenum; and iii) a glutamate dehydrogenase from Aspergillus awamori or Aspergillus niger. The DNA sequence under c) encodes a cleavable linker peptide; as used herein, cleavable linker peptide means a peptide sequence which under certain circumstances allows the separation of the sequences bordering the cleavable linker, for example sequences that are recognized and cleaved by a protease or cleaved after exposure to certain chemicals. In a preferred embodiment, the DNA sequence under c) contains a KEX2 processing sequence. As mentioned above, the desired protein under d) can be in principle any recombinant protein. In a preferred embodiment, the DNA sequence under d) encodes thaumatin; particularly preferred constructions for the preparation of thaumatin include those wherein the DNA sequence encoding thaumatin under d) is the synthetic gene encoding thaumatin II coming from plasmid pThIX, which is disclosed in EP 684312.

Although in the context of the present invention it is preferred, when expressing a desired protein, to use the gdh promoters in fusion constructions, it is also possible to use a gdh promoter to express directly a desired protein. Therefore, it is a further object of the present invention the new DNA constructions that comprise a gdh promoter from a fungus of the genus Aspergillus operatively linked to a DNA sequence encoding the protein that it is desired to express. In a preferred embodiment, the gdh promoter is from

a fungus of the genus Aspergillus with the proviso that it is not from Aspergillus nidulans, more preferably it is from Aspergillus awamori or Aspergillus niger, still more preferably it is from Aspergillus awamori, yet more preferably it is one of the new promoters described above, and more particularly it comprises the nucleotide sequence 1-740 in SEQ ID No. 1.

As will be obvious to those skilled in the art of recombinant DNA technology, all the above DNA constuctions may additionally contain other elements which include, but are not limited to, signal sequences, termination sequences, polyadenylation sequences, selection sequences, sequences that allow the replication of the DNA, etc. There is no limitation on the number and nature of these additional sequences and any of the known sequences for exerting these functions can in principle be used in the constructions according to the present invention. For example, as a signal sequence functional as a secretory sequence we can mention the signal sequences from glucoamylase, α-amylase and aspartyl proteases from Aspergillus awamori, Aspergillus niger, Aspergillus oryzae and Aspergillus sojae, signal sequences from cellobiohydrolase I, cellobiohydrolase II, endoglucanase I and endoglucanase III from Trichoderma species, signal sequences from glucoamylase from Neurospora and Humicola species and the signal sequence from the protein B2 from Acremonium chrysogenum. In general it is preferred to use as signal sequence those derived from proteins secreted by the filamentous fungus used as expression host to express and secrete the recombinant protein or, in case fusion constructions are used, also those derived from the protein used as carrier protein. A termination sequence is a nucleotide sequence which is recognized by the expression host to terminate transcription. Examples include the terminators from the A. nidulans trpC gene, the A. awamori, A. niger, A. oryzae or A. sojae glucoamylase gene, the A. awamori, A. niger, A.

oryzae or A. sojae α-amylase genes and the Saccharomyces cerevisiae cyc1 gene. A selection sequence is a sequence useful as selection marker to allow the selection of transformed host cells. In principle any known selection marker for the filamentous fungus that is intended to be used as host can be employed. Examples of such selection markers include genes confering resistance to a drug such as an antibiotic (e.g. hygromycin or phleomycin) as well as auxotrophic markers such as argB, trpC, niaD and pyrG. A polyadenylation sequence is a nucleotide sequence which when transcribed is recognized by the expression host to add polyadenosine residues to transcribed mRNA. Examples include the polyadenylation sequences from the A. nidulans trpC gene, the A. awamori, A. niger, A. oryzae or A. sojae glucoamylase genes and the Mucor miehei carboxyl protease gene.

The present invention also relates to the filamentous fungus cultures capable of producing a recombinant protein that have been transformed with plasmids that contain any of the DNA constructions mentioned above. Examples of species of filamentous fungi that may be used as expression hosts include the following genera: Aspergillus, Trichoderma, Neurospora, Penicillium, Acremonium, Cephalosporium, Achlya, Phanerochaete, Podospora, Endothia, Mucor, Fusarium, Humicola, Cochliobolus, Rhizopus and Pyricularia. Particularly preferred are those cultures wherein the filamentous fungus is selected from a fungus of the genus Aspergillus, and more preferably it is selected from Aspergillus awamori, Aspergillus niger, Aspergillus oryzae, Aspergillus nidulans or Aspergillus sojae. In another preferred embodiment, the recombinant protein produced is thaumatin.

A further object of the present invention is to provide a process for producing a recombinant protein in a filamentous fungus that comprises the following steps: a) preparation of

an expression plasmid that contains a DNA construction as defined above; b) transformation of a strain of filamentous fungus with said expression plasmid; c) culture of the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneously; and d) depending on each case, separation and purification of the desired protein from the fermentation broth. Preferred is the process wherein the recombinant protein produced is thaumatin.

The accompanying examples describe the identification and isolation of the glutamate dehydrogenase A gene and its promoter region from Aspergillus awamori. This was achieved using a probe from Neurospora crassa. The selection of a suitable DNA fragment from the glutamate dehydrogenase gene in Neurospora crassa to be used as a probe to get the homologous gene in Aspergillus awamori is not, however, straightforward. In this case, there were no clear homology sequences that could be detected, and therefore what was used was a 2.6 kb BamHI fragment that contained the Neurospora crassa gdh gene. This is a large fragment of DNA, and is certainly not the optimal size fragment. Ideally, one wants to use as a probe a highly homologous fragment of DNA, no more than 200-300 bp long. Here a much larger fragment (2600 bp) with undefined homology was used. Yet the present inventors managed to clone a sequence that was later on proven to be the gdh from Aspergillus awamori.

The accompanying examples also describe the application of the above described novel promoters and DNA constructions to the expression of the recombinant protein thaumatin in the filamentous fungus Aspergillus awamori. As shown in these examples, and as illustrated graphically in Figure 12, the expression system of the present invention offers several advantages over the prior art systems. On the one hand, it allows to reach concentrations of expressed protein of about

100 mg/l, which are one order of magnitude higher than the best described (for example, using the process described in EP 684312, concentrations of about 5-10 mg/l are attained; see I. Faus et al., Appl. Microbiol. Biotechnol., 1998, vol. 49, pp. 393-398). On the other hand, for a same carrier protein and a same fermentation time, the use of the promoter of the present invention leads to higher concentrations of expressed protein. And last but not least, with the constructions of the present invention it is possible to use a more economical nitrogen source (ammonium sulfate) than the one that is commonly used (asparagine).

DEFINITIONS

The term "promoter" means a DNA sequence operative in a filamentous fungus capable of promoting transcription of a coding region when operatively associated therewith.

The term "recombinant protein" means a protein that is not expressed under standard normal conditions by the host, and that is only expressed by the host as a result of the introduction into said host of a DNA sequence that allows for the expression of said recombinant protein. This recombinant protein can be fungal or non-fungal, and it can even be found in the non-recombinant host.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, parts A, B and C. Schematic representation of the steps involved in the construction of the B2KEX expression cassette.

Figure 2. Restriction map of a 28.7 kb region of A. awamori DNA including the gdhA gene. Map of phages FAN1 and FAN2. Thick lines indicate the overlapping zone between the two

phages containing the gdhA gene. pB10, pB5.5 and PB1.7 indicate the DNA fragments subcloned in the corresponding plasmids. B = BamHI, S = Sal I.

Figure 3. Restriction map of the 2.1 kb XbaI-BamHI fragment from pB5.5 plasmid that was sequenced. The 3' end of the gdhA gene was contained in the left region of the insert in pB1.7. B = BamHI, E = EcoRI, EV = EcoRV, P = PstI, S = SalI, X = XbaI.

Figure 4, parts A and B. Alignment of the deduced amino acid sequences of NADP-specific glutamate dehydrogenases of A. awamori, A. nidulans (Genebank accession number P18819), N. crassa (P00369), S. cerevisiae (P07262), S. occidentalis (P29507), A. bisporus (P54387), S. typhimurium (P15111), E. coli (P00370) and C. glutamicum (P31026). Identical amino acids are shadowed. Motifs a-i with several consecutive conserved residues are overlined.

Figure 5. Complementation of the gdhA mutation in two strains of A. nidulans with the gdhA gene of A. awamori. Part A: 1, A. nidulans A686 mutant; 2, transformant A686-4; 3, transformant A686-6; 4, transformant A686-7. Part B. 1, A. nidulans A699 mutant; 2, transfomant A699-2; 3, transformant A699-3; and 4, transformant A699-4.

Figure 6. Primer extension identification of the 5' end of the gdhA gene transcript. One protected band (arrow) is observed in the lane corresponding to the extension reaction (lane Pe). G, A, T, C lanes correspond to the sequencing reactions of M13 phage from the -40 primer.

Figure 7. Northern blot analysis of the transcripts of the gdhA and ß-actin genes. A: hybridization with a probe internal to the gdhA gene (0.694 kb PvuII fragment). B: hybridization with the ß-actin gene of A. nidulans as control.

Figure 8. Slot Blot analysis of the trancript of the A. awamori gdhA gene, during the course of a fermentation in MDFA medium with 1% glucose and 10 mM ammonium sulfate (part A). For comparative purposes, the transcript of the ß-actin gene in the same RNA sample was also studied. Part B: relative level of the expression of the gdhA to the ß-actin gene. Part C: NADP-dependent glutamate dehydrogenase activity in the same cultures from where the mRNAs were extracted.

Figure 9. Slot Blot analysis of the transcript of the A. awamori gdhA gene during the course of a fermentation in MDFA medium with different nitrogen sources (part A). The medium contained ammonium sulfate 10 mM as a control and glutamic acid, glutamine, sodium nitrite, sodium nitrate and asparagine as nitrogen source, all of them at a concentration of 10 mM. The transcript of the ß-actin gene was also studied for comparative purposes. Part B: Relative level of expression of the gdhA to the ß-actin gene.

Figure 10, parts A, B and C. Schematic representation of the steps involved in the construction of the GDH expression cassette.

Figure 11, parts A and B. Schematic representation of the steps involved in the construction of the GPD expression cassette.

Figure 12. Production (expressed as concentratin CT of secreted protein in mg/l) of thaumatin from A. awamori strains TB2b1-44 and TGDTh-4 in fermentor studies. The medium used was MDFA supplemented with the components described below. Empty squares: Strain TB2b1-44; 6.0% sucrose, pH 6.2, fedbatch with asparagine. Empty circles: TB2b1-44, 6.0% sucrose, pH 6.2, fedbatch with ammonium sulfate. Filled triangles: Strain TGDTh-4; 6.0 % sucrose, pH 6.2, fed-batch with ammonium sulfate.

DETAILED DESCRIPTION OF ONE MODE OF CARRYING OUT THE INVENTION

This section describes the application of the new promoter and constructions described in the present invention to the preparation of recombinant thaumatin. The teachings of the examples below can be applied to the expression and production of any other recombinant protein and thus these examples should not be construed as limiting the scope of the present invention in any way.

A: CONSTRUCTS:

The starting point for all of the constructs that have been prepared in the present patent application is plasmid pThIX, which is described in European patent application EP 684312. This plasmid contains: (i) a sulfanilamide resistance marker; (ii) a DNA sequence which encodes a fusion protein comprising in his turn (a) the synthetic gene encoding thaumatin II, (b) a spacer sequence which in turn contains a KEX2 processing sequence, and (c) the complete glucoamylase gene (genomic) of Aspergillus niger; (iii) the signal sequence ("pre") and the "pro" sequence of the glucoamylase gene (glaA) of Aspergillus niger, and finally (iv) the promoter region sequence of the glucoamylase gene (glaA) of Aspergillus niger.

In the context of the present invention three new expression cassettes were prepared, which contained: (i) a drug resistance marker (most of the times it was a phleomycin resistance marker); (ii) a DNA sequence which encodes a fusion protein comprising in his turn (a) the synthetic gene of thaumatin II, (b) a spacer sequence which in turn contains a KEX2 processing sequence, and (c) a cDNA sequence that encodes most of the B2 protein (except sequences in the COOH end) from Acremonium chrysogenum; (iii) the signal sequence of the B2 gene of Acremonium chrysogenum and (iv) three

different promoter regions.

In all the cloning and sub-cloning manipulations described in this patent application, Escherichia coli DH5a served as the recipient strain for high-frequency plasmid transformation. E. coli WK6 was used as host for obtaining single-stranded DNA from pBluescript plasmids for sequencing purposes.

A1. Construction of the expression cassette B2KEX

Protein B2 is an extracellular protease produced by the filamentous fungus Acremonium chrysogenum. This protein is expressed and secreted in the late stages of growth of Acremonium chrysogenum (between 120 and 144 hours after the start of growth).

Plasmid pJE1A (Laboratory of Prof. Juan-Francisco Martín, Universidad de León, León, Spain) contains the promoter region, leader peptide (including the signal sequence) and coding region of the B2 gene from Acremonium chrysogenum. The gene itself has 1298 base pairs and two introns. These two introns are not present in the sequence that has been subcloned in pJE1A, since these subcloned sequences were obtained from a cDNA. Upstream from the ATG start point of translation there is a promoter region of 477 base pairs. When Acremonium chrysogenum is grown in a defined medium which contains sucrose and glucose as carbon sources and asparagine as nitrogen source, the gene is expressed at its highest levels between 72 and 96 hours of growth.

The steps involved in the construction of the B2KEX cassette are detailed in Figure 1 (parts A-C). Plasmid pJE1A was digested sequentially with BamHI and NcoI, releasing a 560 bp fragment that was purified from a 0.8% agarose gel. This fragment contains most of the coding region of the B2 gene, but excludes the active center of the protein. Similarly, plasmid pJL43b (J.L. Barredo, Ph.D. Thesis, Universidad de

León, León, Spain) was also digested with BamHI and NcoI, releasing a large fragment (3740 bp), which was purified from a 0.8% agarose gel. This fragment was ligated with the 560 bp BamHI-NcoI fragment from pJE1A, yielding plasmid p43bB2CT (4300 bp).

Plasmid p43bB2CT was digested with NcoI, treated with the Klenow fragment of DNA polymerase I (in order to obtain blunt ends) and then digested with StuI, yielding a fragment of 3874 bp that was also purified from a 0.8% agarose gel. The single-stranded oligonucleotides ThS1 and ThS2 (sequences shown below) where used, using plasmid pThIX as a template, to amplify by polymerase chain reaction (PCR) the KEX2-like and thaumatin sequences present in pThIX. The first 18 nucleotides present in ThS1 correspond to the KEX2-like sequence.

ThS1: 5'- CGA ATG AAA AGG AAA AGG ATGGCCACCTTCGAG - 3'
Arg Met Lys Arg Lys Arg

ThS2: 5'- TTA TTA GGC GGT GGG GCA - 3'

A 655 bp DNA fragment was obtained by PCR using plasmid pThIX as the template and ThS1 and ThS2 oligonucleotides as primers. This DNA fragment was ligated with the previously obtained fragment from p43bB2CT, yielding plasmid p43bB2CTTh. This plasmid (aprox. 4530 bp) contains part of the B2 protein gene fused to a KEX-2 sequence and to the synthetic gene encoding thaumatin II. The transcription termination signal present in this construct is the terminator sequence from the cyc1 gene of Saccharomyces cerevisiae.

Plasmid p43bB2CTTh was digested with BamHI, treated with calf intestinal alkaline phosphatase (CIP) and purified from a 0.8% agarose gel. A 900 bp BamHI-BamHI fragment from pJE1A was also isolated. Subsequent ligation of these two DNA fragments generated plasmid pB2KEX (5430 bp). The 900 bp

BamHI-BamHI fragment from pJE1A contains the B2 gene promoter sequence (477 bp), the leader peptide sequence (318 bp) and 107 bp of the amino terminal sequence of the B2 gene.

Plasmid pB2KEX was then digested with XbaI, treated with the Klenow fragment of DNA polymerase I (in order to obtain blunt ends) and then digested with SalI, yielding a fragment of 2400 bp that was purified in a 0.8% agarose gel. Plasmid pJL43b was digested with HindIII, also treated with the Klenow fragment of DNA polymerase I, and then digested with XhoI. A fragment of 4500 bp was purified as before. Finally, the two gel-purified fragments described above were ligated, generating plasmid pB2KTh (6900 bp; Fig. 1C).

On the final sub-cloning step, both plasmids pB2KTh and pJL43b1 were digested with SacI and StuI, yielding fragments of 5714 and 1305 bp, respectively, which were purified in a 0.8% agarose gel. These two fragments were then ligated, thus obtaining plasmid pB2KThb1 (7020 bp; Fig. 1C). Plasmid pJL43b1 is a derivative of plasmid pJL43b, where the promoter that drives expression of the phleomycin resistance gene (PpcbC from Penicillium chrysogenum) was substituted by the glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter from Aspergillus nidulans (P. Punt et al., Gene 1990, vol. 93, pp.101-109).

This plasmid contains a cassette to express thaumatin that comprises: (i) a phleomycin resistance marker; (ii) a DNA sequence which encodes a fusion protein comprising in his turn (a) the synthetic gene of thaumatin II, (b) a spacer sequence which in turn contains a KEX2 processing sequence, and (c) a cDNA sequence that encodes most of the B2 protein (except sequences in the COOH end) from Acremonium chrysogenum; (iii) the signal sequence of the B2 gene of Acremonium chrysogenum and (iv) the promoter region of the B2 gene of Acremonium chrysogenum. In this particular construct, expression of the phleomycin resistance gene

(phleo) is driven by the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene from Aspergillus nidulans.

A2. Construction of the expression cassette GDHTh

A.2.1. Cloning of a DNA fragment of Aspergillus awamori containing the gdhA gene.

A. awamori ATCC 22342 was used as the source of DNA and RNA. A. nidulans mutants A686 (gdhA1, yA2, methH2, galA1) and A. nidulans A699 (gdhA1, biA1) (J.R. Kinghorn, J.A. Pateman, J. Gen. Microbiol. 1973, vol. 78, pp. 39-46) were obtained from the Fungal Genetics Stock Center, and were used for complementation studies with the gdhA gene from A. awamori. The partial glutamate auxotrophy of these two strains was confirmed by growth on media with glutamic acid or high ammonium sulfate concentrations (100 mM) as nitrogen source. Both gdhA mutants grow very poorly under high ammonium sulfate concentrations but show normal growth when glutamic acid is used as nitrogen source. E. coli NM539 served as host for Lambda GEM12 (Promega Co., Wis) phage derivatives.

Filamentous fungi were routinely maintained on solid Power sporulation medium (F. Fierro et al., Appl. Microbiol. Biotechnol. 1996, vol. 43, pp. 597-604) at 30°C for 3 days. A. awamori and A. nidulans seed cultures in CM medium (containing 20 g/l malt extract; 5 g/l yeast extract; 5 g/l glucose) were inoculated with 10^6 spores/ml and grown at 28°C in a rotary G10 incubator (New Brunswick Scientific, New Brunswick, N.J.) for 48 h. For gdhA transcript isolation and characterization studies, A. awamori cultures in MDFA medium (Y.Q. Shen et al., J. Antibiot. 1984, vol. 37, pp. 503-511) were incubated with a 15 % seed culture and grown at 30°C for 48-72 h in a rotary shaker, as described above.

A.2.1.1. Aspergillus awamori genomic library

A genomic library of total DNA of A. awamori ATCC 22342 was constructed in a Lambda GEM12 phage vector. Total DNA was extracted and partially digested with Sau3AI to obtain DNA fragments of between 17 and 23 kb. This DNA was purified by sucrose-gradient centrifugation, ligated to Lambda GEM12 phage arms, and packaged in vitro using a Gigapack III Gold packaging system (Stratagene) resulting in a total of $8x10^4$ recombinant phages.

In the next step, and using as probe a 2.6 kb BamHI fragment containing the gdhA gene of Neurospora crassa (J.H. Kinnaird, J.R.S. Fincham, Gene 1983, vol. 26, pp. 253-260), two phages, FAN1 and FAN2, that gave a clear hybridization signal were isolated and purified by three rounds of infection. Restriction mapping of these two phages showed that they overlap in 7.2 kb. The total DNA region cloned in the two phages extended for 28.7 kb.

BamHI fragments of 1.7, 5.5 and 10 kb were subcloned in pBluescript KS+ plasmid, giving rise to plasmids pB1.7, pB5.5 and pB10, as shown in Figure 2. They were then sequenced by generating ordered sets of deletions with the Erase-a-base system (Promega Co., Wis.) by digestion with exonuclease III from appropriate ends, followed by removal of single-stranded DNA with S1 exonuclease. Sequencing of fragments of the gdhA gene was performed by the dideoxynucleotide chain termination method. For sequencing the cDNA clones containing the intron-exon junctions, reactions were performed with 90 ng of dsDNA using the GeneAmp PCR 2400 system coupled to the ABI-PRISM 310 automatic sequencer (Perkin Elmer). Computer analysis of nucleotide and amino-acid sequences were made with the DNASTAR software (DNASTAR, Inc., UK).

Initial sequencing showed that an open reading frame (ORF1) occurred in the right end of the 5.5 kb insert of pB5.5 extending into the left region of the 1.7 kb BamHI fragment

of pB1.7, as shown in Figure 3. The 5.5 kb and 1.7 kb BamHI fragments were mapped in detail.

A 2.1 kb XbaI-XbaI fragment corresponding to the right end of plasmid pB5.5 was subcloned in pBluescript SK+ plasmid, creating plasmid pBSGh. More specifically, this 2.1 Kb XbaI-XbaI fragment was generated by digesting pB5.5 at an internal XbaI site and at a second XbaI site in the polylinker of pBSKS+ (and close to the BamHI site shown in Fig. 3).

A region of 2570 nt was sequenced in both strands by the dideoxynucleotide chain termination method. This region contained ORF1 (1380 bp), which started at an ATG located 740 bp downstream from the left end of the insert in pBSGh and extended until the end of the 5.5 kb BamHI fragment, with 60 additional bp into the adjacent 1.7 kb fragment. ORF1 was preceeded by a 740 nucleotide region that contained the necessary signals required for transcription initiation and regulation (see SEQ. ID No. 1).

ORF1 contained two putative introns at positions 785-850 and 1414-1471 (following the numbering in SEQ ID No. 1) that showed lariat and 5' and 3' splicing sequences similar to those of other fungal introns (D.J. Ballance, Yeast 1986, vol. 2, pp. 229-236). The presence of the two introns was confirmed by sequencing the DNA regions corresponding to introns I and II obtained by PCR from a A. awamori cDNA library using as primers oligonucleotides I_A and I_B for intron I, and II_A and II_B for intron II (sequences shown below).

cDNA for these experiments was obtained from total RNA extracted as described above, from mycelia grown for 48 h in MDFA medium. The first and second cDNA strands were synthetized using a cDNA synthesis kit from Stratagene (La Jolla, Ca). This cDNA was used for PCR amplification of the fragments containing the exon-exon junctions by the following program: 1 cycle at 94ºC for 5 min, 50ºC for 1 min, 72ºC for

1 min followed by 30 cycles at 94ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min and finally one cycle at 72ºC for 8 min.

Oligonucleotides:

I_A 5' ATG TCT AAC CTT CCT CAC 3'
I_B 5' ACC CTT ACC ACC ACC CAT 3'
II_A 5' CGC TTC TGT GTT TCC TTC 3'
II_B 5' GTA CTT GAA CTT GTT GGC 3'

A.2.1.2. ORF1 encodes a putative NADP-dependent glutamate dehydrogenase

ORF1 encoded a protein of 460 amino acids (see SEQ ID No. 2) with a deduced molecular mass of 49.4 kDa and a pI value of 5.62. Comparison of the protein encoded by ORF1 with other proteins in the SWISS-PROT data base showed that the encoded protein has a high homology with NADP-dependent glutamate dehydrogenases of A. nidulans (84.7% of identical amino acids), N. crassa (74.4% identity), Saccharomyces cerevisiae (66.5% identity) and Schwanniomyces occidentalis (66.9% identity), as shown in Figure 4. The homology is extensive throughout the entire protein. All these proteins are NADP-dependent glutamate dehydrogenases that catalyze the reductive amination of α-ketoglutarate, in the presence of ATP, to form L-glutamate. The protein encoded by ORF1 contains nine conserved motifs when compared with other fungal and yeast glutamate dehydrogenases. One of the conserved domains (amino acids 108-121) corresponds to a region implicated in the catalytic mechanism of the enzyme. The consensus sequence of this region is [LIV]-X(2)-G-G-[SAG]-K-X-[GV]-X(3)-[DNS]-[PL] (PROSITE PS00074). The lysine residue K^{114} located in the glycine-rich region GGGK^{114}GG corresponds to the lysine in the active center of Glu/Leu/Phe/Val (GLFV) dehydrogenases. Therefore, following standard fungal gene nomenclature, the gene encoded by ORF1 was named gdhA.

A.2.1.3. The cloned gene complements A.nidulans gdhA mutants

A. nidulans A686 and A699 strains were transformed by a known method (M.M. Yelton et al. Proc. Natl. Acad. Sci. USA 1984, vol. 81, pp. 1470-4) with plasmid pGDHaw (7.1 kb), which contains the A. awamori gdhA gene in a 2570 bp XbaI-XbaI fragment. This fragment contains also an upstream promoter region of 740 bp and a 322 bp region downstream from ORF1 (gdhA gene). The 2570 bp XbaI-XBaI fragment was inserted into the XbaI site of the fungal vector p43gdh, which contains the phleomycin resistance marker under control of the A. awamori gdhA promoter as shown later in this patent application.

Seven transformants of A. nidulans A686 with the A. awamori gdhA gene and 15 transformants of A. nidulans A699 were analyzed on minimal medium supplemented with different concentrations (10, 50 and 100 mM) of ammonium sulfate as nitrogen source, and their growth was compared with that of wild type A. nidulans. As a control, growth was also tested on medium containing 10 mM glutamic acid. As shown in Figure 5, the untransformed A. nidulans mutants A686 and A699 grow very poorly in plates with 100 mM ammonium sulfate, whereas three randomly selected transformants grow very well in this medium. The residual growth of A. nidulans gdhA mutants A686 and A699 in ammonium sulfate as nitrogen source is known (J.R. Kinghorn, J.A. Pateman, Heredity 1973, vol. 31, pp. 427) and is due to the presence of a second glutamate dehydrogenase activity that allows partial growth of these mutants.

A.2.1.4. Glutamate dehydrogenase activity in the transformants

Nicotinamide adenine dinucleotide phosphate (NADP)-specific glutamate dehydrogenase (NADP-GDH) activity was assayed by

following the reductive amination of α-ketoglutarate in the presence of ammonium and NADPH and expressed as units of enzyme activity per mg protein. The initial reaction velocity was estimated from the change in optical density at 340 nm in a Hitachi U-2001 spectrophotometer. One unit of glutamate dehydrogenase was defined as the activity that catalyzes the oxydation of one nanomol of NADPH per minute.

To confirm the complementation results, the NADP-dependent glutamate dehydrogenase activity was measured in the A. nidulans gdhA mutants A686 and A699, and in three randomly selected transformants complemented with the A. awamori gdhA gene. Results are shown in Table 1 and they clearly indicated that while the glutamate dehydrogenase activity in strains A686 and A699 was clearly below the detection levels, significant levels of glutamate dehydrogenase activity were obtained in the transformants with the A. awamori gdhA gene, particularly at 24 and 48 h of growth. Some of the transformants, like A699-4, showed relatively high levels of glutamate dehydrogenase activity, perhaps due to integration of more than one copy of the gdhA gene in the genome of this transformant.

Table 1: NADP-dependent glutamate dehydrogenase activity (U/mg of protein), in the A. nidulans gdhA mutants A686 and A699, and in three transformants of each of these mutants with the A. awamori gdhA gene.

strain	t = 24 h	t = 48 h	t = 72 h
A. Awamori	550	0	0
A686	0	0	0
A686-4	350	280	100
A686-6	340	200	80
A686-7	310	160	90
A699	0	0	0

A699-2	270	240	100
A699-3	410	420	400
A699-4	500	670	580

A.2.1.5. Characterization of the promoter region of the gdhA gene

Analysis of the nucleotide sequence upstream from the ATG translation initiation codon revealed the presence of GTATA, CTATA and TCAATC sequences at positions -316, -61 and -17, respectively, with respect to the translation initiation codon, which may correspond to putative TATA and CAAT boxes involved in regulation of gene expression (see SEQ ID No. 1).

Identification of the transcription start point was performed by "primer extension" with 2 µg of mRNA obtained from mycelia grown in MDFA for 48 h, as shown in Figure 6.

Primer extension analysis using as primer the oligonucleotide "Pe" 5'-GGGGTTCTTCTGGAAGAGGGT-3' (corresponding to the nucleotide sequence 70 bp downstream from the ATG) revealed a single band in the extension reaction (Fig. 8). The 5'-end of the mRNA corresponds to a thymine (T) located 86 bp upstream of the ATG initiation codon.

A.2.1.6. The gdhA gene is transcribed as a monocistronic transcript of 1.7 kb, and its expression is regulated by nitrogen.

In order to perform expression studies, total RNA of A. awamori was obtained by the phenol-SDS method from mycelia grown for 12, 24, 48, 60 or 72 h in MDFA medium with 55.5 mM glucose and 10 mM ammonium sulfate as carbon and nitrogen sources, respectively. For nitrogen regulation studies, the MDFA base medium (without ammonium sulfate) was supplemented with glutamic acid, L-glutamine, sodium nitrite, sodium

nitrate and L-asparagine at 10 mM final concentrations.

For Northern analysis, total RNA (5 µg) was run on a 1.2% agarose-formaldehyde gel. The gel was blotted onto a nylon filter (NYTRAN 0.45; Schleicher and Schuell) by standard methods. The RNA was fixed by UV irradiation using an UV-Stratalinker 2400 lamp (Stratagene, La Jolla, Calif.).

For slot blotting, the RNA (5 µg) was loaded on a filter (NYTRAN 0.45) by vacuum in a Bio-Dot SF Microfiltration apparatus (Slot Blotting, Bio-Rad). The RNA was fixed by UV irradiation as above. The filters were pre-hybridized for 3 h at 42°C in 50% formamide, 5 x Denhardt's solution, 5 x SSPE, 0.1% SDS, 500 µg of denatured salmon-sperm DNA per ml, and hybridized in the same buffer containing 100 µg of denatured salmon-sperm DNA per ml at 42°C for 18 h, using as probe an internal DNA fragment (0.694 kb PvuII) of the A. awamori gdhA gene. The filters were washed once in 2 x SSC, 0.1% SDS at 42°C for 15 min, once in 0.1 x SSC, 0.1% SDS at 42°C for 15 min, and once more in 0.1 x SSC, 0.1% SDS at 55°C for 20 min and then autoradiographed with Amersham X-ray film. mRNA was purified from total RNA by using the Poly(A) Quick mRNA isolation kit (Stratagene, La Jolla, Calif.).

Northern analysis of the transcription of the gdhA gene revealed that it is strongly expressed as a 1.7 kb transcript (mRNA) with a size slightly larger than that of the ß-actin gene mRNA, as shown in Figure 7. Since ORF1 contains 1380 nt, this size of the transcript indicates that the gdhA gene is expressed as a monocistronic transcript.

Since the same amount of total RNA was used in all lanes of Fig. 7, it was concluded that the gdhA steady state transcript levels in the cell are higher than those of the ß-actin gene (arrows) indicating that the glutamate dehydrogenase A is expressed from a very efficient promoter.

To determine the pattern of expression of the gdhA gene during the time-course of growth of A. awamori, gdhA hybridizing RNA was compared to ß-actin hybridizing RNA in MDFA medium with ammonium sulfate (Figure 8A) and expressed as the ratio of counts in the gdhA-hybridizing band to the ß-actin hybridizing counts (Figure 8B). Results indicate that expression of both genes (gdhA and ß-actin) is associated with the growth of A. awamori but whereas low steady state levels of ß-actin mRNA remained in the cells until 96 hours of growth, the levels of glutamate dehydrogenase mRNA decreased drastically after 48 hours.

The glutamate dehydrogenase enzymatic activity detected when A. awamori is grown in MDFA medium with ammonium sulfate (10 mM) as nitrogen source at different times of the culture is shown in Fig. 8C. There is a sharp decrease in glutamate dehydrogenase activity between 24 and 48 h after start of growth, which is in good agreement with the decrease in transcript levels at this time of the culture, as shown in Fig. 8B.

Since glutamate dehydrogenase plays a central role in nitrogen utilization by A. awamori, it was also of interest to study if expression of gdhA was regulated by different nitrogen sources. As shown in Figure 9, very high gdhA transcript (mRNA) levels were obtained in media containing NH_4^+, or asparagine as sole nitrogen sources. Glutamic acid repressed transcription of the gdhA gene, whereas intermediate levels of expression (normalized with respect to the ß-actin gene) were observed in media that contained nitrate, glutamine or nitrite as nitrogen source. These results show that the NADP-dependent glutamate dehydrogenase is subject to a strong nitrogen regulation at the transcriptional level.

The glutamate dehydrogenase activity in 24-hour cultures grown in MDFA medium containing different nitrogen sources,

all at a concentration of 10 mM, is shown in Table 2. The highest activity (per ml of culture) was observed in cultures with NH_4^+ or asparagine as nitrogen sources. Moreover, these two nitrogen sources favoured a strong growth of A. awamori. When the results were expressed per mg of protein in the cell extracts, the highest specific activity was observed in MDFA medium with nitrate as the sole nitrogen source. This is due to the fact that in the presence of nitrate, A. awamori grows very slowly. The lowest activity was observed in MDFA medium with glutamate as nitrogen source, confirming the results observed previously at the transcription level.

Table 2: NADP-dependent glutamate dehydrogenase activity in A. awamori cultures grown for 24 h in MDFA medium supplemented with different nitrogen sources.

Nitrogen source (10mM)	Total Activity (U/ml)	Specific Activity (U/mg protein)
ammonium	1450	800
glutamic acid	330	280
glutamine	1100	600
nitrite	990	660
nitrate	1150	1680
asparagine	1300	720

A.2.2. Construction of the expression cassette GDHTh

Once the promoter region of the gdhA gene was located, a thaumatin expression cassette similar to the one described previously was constructed. Plasmid pBSGh was used as a template to obtain a 750 bp DNA fragment corresponding to the promoter region of the gdhA gene. This fragment was obtained by DNA amplification using the oligonucleotides gdh1 and gdh2 and the Pfu enzyme (Stratagene).

gdh1: 5' - TTTT GTCGAC TTG CGA CGG CGT ATT GCT - 3'
Sal I

gdh2: 5' - TTTT CCATGG TCT GAA GGG GAG GAT TGA - 3'
Nco I

This amplified DNA fragment was digested with SalI and NcoI and purified in a 0.8% agarose gel.

Plasmid pJL43 (a derivative of pJL43b, Dr. José Luis Barredo, Ph.D. Thesis, Universidad de León, León, Spain) was digested with SalI and NcoI and a large fragment (3740 bp) was purified in a 0.8% agarose gel. This DNA fragment was then ligated with the SalI-NcoI fragment previously amplified, yielding plasmid p43gdh (4500 bp), where the pcbC promoter from Penicillium chrysogenum has been replaced by the gdhA promoter from Aspergillus awamori.

In the next step, plasmid p43gdh was digested with NcoI, treated first with the Klenow fragment of DNA polymerase I and then with calf-intestinal phosphatase (CIP). In paralell, a fragment of 1140 bp containing the B2 protein gene was amplified via the PCR technique, using plasmid pJE1A as the template and oligonucleotides NTB2b and CTB2b as primers (sequences given below). This 1140 bp fragment was digested with BamHI and then treated with the Klenow fragment of DNA polymerase I. From this reaction mix a 425 bp DNA fragment containing the amino terminal sequences of the B2 gene was purified from a 1.0 % agarose gel. This fragment of DNA was ligated by blunt-end ligation to the fragment of DNA from p43gdh previously described, resulting in plasmid p43gdhB2, where the BamHI site that is shown in Figure 10 has been regenerated. This plasmid is 4925 bp long and contains the gdhA promoter fused "in frame" to the amino terminal portion of the B2 gene.

The next step in the construction of the complete expression cassette was the addition of the second portion of the B2 gene, the KEX2 sequence and the synthetic thaumatin II gene. For this part of the work, plasmid pB2KEX was used.

pB2KEX was sequentially digested with XbaI, treated with the Klenow fragment from DNA polymerase I and finally digested with BamHI. A fragment of 4637 bp was purified from a 0.8% agarose gel. In paralell, plasmid p43gdhB2 was sequentially digested with SalI, treated with the Klenow fragment from DNA polymerase I and finally digested with BamHI. A fragment of 1173 bp was purified from a 0.8% agarose gel. The ligation of these two fragments yielded plasmid pGDHTh (5810 bp), where a new SalI site was created. This allows for the excision of the complete GDHTh cassette as a 2670 bp SalI-SalI fragment.

Starting with plasmid pGDHTh, two new plasmids were constructed. The first one was p43GDTh, constructed as follows. Plasmid pJL43 was linearized by digestion with SalI and ligated to a 2170 bp SalI-DraI fragment from pGDHTh (see Fig. 10, part B).

Similarly, plasmid pGD71 was constructed as follows: plasmid pAN7-1 (P.J. Punt et al., J. Biotecnol. 1990, vol. 17, pp. 19-34) was sequentially digested with XbaI, treated with the Klenow fragment from DNA polymerase I, and finally digested with HindIII, and purified from a 0.8% agarose gel. In paralell, plasmid pGDHTh was digested with Ec1136II (or SacI*, a variant of SacI from Fermentas that recognizes the standard SacI restriction site but leaves a blunt end), HindIII and DraI. A fragment of 2175 bp was purified from an agarose gel. Ligation of these two fragments yielded plasmid pGD71 (see Fig. 10, part C).

Plasmids p43GDTh and pGD71 contain a cassette to express thaumatin that comprises: (i) a DNA sequence which encodes a fusion protein comprising in his turn (a) the synthetic gene

of thaumatin II, (b) a spacer sequence which in turn contains a KEX2 processing sequence, and (c) a cDNA sequence that encodes most of the B2 protein (except sequences in the COOH end) from Acremonium chrysogenum; (ii) the signal sequence of the B2 gene of Acremonium chrysogenum, (iii) the promoter region from the Aspergillus awamori glutamate dehydrogenase A gene, and (iv) a drug resistance gene that can be used as a transformation marker. Plasmid p43GDTh has the phleomycin resistance gene (phleo) driven by the the pcbC promoter from Penicillium chrysogenum. Plasmid pGD71 contains the hygromycin B resistance gene driven by the glyceraldehyde-3-phosphate dehydrogenase promoter from Aspergillus nidulans.

A.3. Construction of the expression cassette GPDTh

The expression cassette GPDTh is similar to the expression cassette B2KEX, except that the B2 promoter from Acremonium chrysogenum has been replaced by the promoter from the glyceraldehyde-3-phosphate dehydrogenase (named "gpd" from now on) gene from Aspergillus nidulans.

The complete promoter region of the gpd gene is present in plasmid pAN52-1 (P.J. Punt et al., J. Biotecnol. 1990, vol. 17, pp. 19-34). A SacI-NcoI fragment (880 bp) from pAN52-1 has been subcloned, generating pJL43b1.

Plasmid pJL43b1 was digested with NcoI and treated first with the Klenow fragment of DNA polymerase I and then with calf-intestinal phosphatase (CIP), as shown in Figure 11. In parallel, a 1140 bp fragment of DNA was obtained by DNA amplification using the PCR technique, using pJE1A as template and oligonucleotides NTB2b and CTB2b as primers. This fragment of DNA was digested with BamHI and treated with the Klenow fragment from DNA polymerase I, yielding a fragment of 425 bp that was purified from a 0.8% agarose gel. The final ligation reaction yielded plasmid pb1B2 (see Fig. 11).

NTB2b: 5' - ATG CGT GCT GCT ACT CTC - 3'
CTB2b: 5' - CTG GCC GTT GTT GAT GAG - 3'

As with the GDHTh cassette, the next step in the construction of a complete expression cassette was the addition of the second portion of the B2 gene, the KEX2 sequence and the synthetic thaumatin II gene. For this part of the work, plasmid pB2KEX was once again used.

pB2KEX was sequentially digested with XbaI, treated with the Klenow fragment from DNA polymerase I and finally digested with BamHI. A fragment of 4637 bp was purified from a 0.8% agarose gel. In paralell, plasmid pb1B2 was sequentially digested with BamHI and Ec1136II (or SacI*) (leaves blunt ends), and a 1300 bp fragment was purified from a 0.8% agarose gel. The ligation of these two fragments yielded plasmid pGPDTh (5800 bp).

In the next step, the GPDTh cassette was isolated from pGPDTh by digestion with Ec1136II (or SacI*), HindIII and DraI, yielding a DNA fragment 2800 bp long. In parallel, plasmid pB2KThb1 was sequentially digested with BamHI, treated with the Klenow fragment from DNA polymerase I and finally digested with HindIII. A 4500 bp fragment was isolated from a 0.8% agarose gel. The plasmid resulting from the ligation of these two fragments was named pGPThb1.

This plasmid contains a cassette for the expression of thaumatin that is identical to the expression cassette B2KEX except that the promoter from the B2 gene of Acremonium chrysogenum has been replaced by the promoter from the gpd gene from Aspergillus nidulans.

B. Strains used and transformation protocol

Aspergillus awamori strain NRRL312 was obtained from the

American Type Culture Collection (ATCC). Using standard mutagenesis techniques with nitrosoguanidine (NTG), a derivative of this strain was obtained, and was named LpR66. This mutant strain secretes into the growth medium an inactive exoprotease aspergillopepsin A (named "pepA" from now on). In all of the transformation experiments that are described below the strain that was used was Aspergillus awamori strain LpR66.

The three expression cassettes that have been described previously were used to transform Aspergillus awamori strain LpR66.

In all single transformation experiments, the antibiotic phleomycin was used as the selection marker. Strain LpR66 can grow in plates that contain 20 µg/ml of phleomycin. Therefore, all transformants were selected in plates with 25 µg/ml of the antibiotic. The regeneration medium that was used is TSAS, which contains 30 g/l of Triptone-Soja (Difco), 103 g/l of sucrose and 1.5% agar (Difco).

The transformation protocol was similar to the one described by Yelton (see above) with some modifications. A plate containing Power medium was inoculated with 10^7 spores. This plate was incubated for 72 hours at 30ºC, at which point the spores were scraped from the plate and were inoculated in 100 ml of CM medium (500 ml shake flask). Incubation was for 16-18 hours at 250 rpm and 28ºC. The mycelium obtained from this growth was filtered through a 30 µm nylon filter (Nytal) and washed with 10 mM sodium phosphate buffer (pH 5.8) which also contained 0.6 M magnesium sulfate. One gram of mycelium was re-suspended in "protoplast buffer" (10 mM sodium phosphate buffer (pH 5.8) which also contained 1.2 M magnesium sulfate). An equal volume of buffer containing the enzyme "Lysing" (Sigma) was added, yielding a final concentration of 3 mg/ml of the enzyme. The mycelium solution was left to incubate for 3-4 hours at 100 rpm and 30ºC until protoplasts

were formed. Protoplast formation was monitored by visual inspection using a light microscope. Protoplasts were filtered, washed and finally resuspended in STC solution, to a final concentration of 10^8 protoplasts/ml.

100 µl of protoplast solution was mixed with 10-20 µg of DNA and left in ice for 20 minutes. After this time interval, 500 µl of PTC were added, and left at room temperature for another 20 minutes. Then, 600 µl of STC medium were added and the transformation mix was aliquoted in different test tubes. Finally, the phleomycin antibiotic solution and TSAS medium that contained agar were added. The contents of the tubes were gently homogenized and added to TSAS plates that contained phleomycin. Plates were incubated at 30ºC until the transformants were visualized as individual colonies. When hygromycin B was used as selection marker, a similar protocol was used.

The linearization of all the plasmids that have been described in this work gave a 4-fold increase in the efficiency of transformation as compared to transformations performed with plasmids that had not been linearized. Therefore, in most transformation experiments the plasmids were used linearized.

Several transformants were obtained and analyzed. Initial screens were performed in plates containing 25 µg/ml of phleomycin. Confirmation screens were then performed using phleomycin concentrations as high as 200 µg/ml.

Transformants were analyzed by PCR to detect whether the thaumatin II gene had been incorporated into their genome essentially as described (cf. EP 684312). Those transformants that were positive were then further analyzed for expression of thaumatin by immunoblot analysis and ELISA (enzyme-linked immunoassay) also as described (cf. EP 684312).

C: Recombinant strains that produce thaumatin

C.1. Materials and methods

C.1.1. Culture media

CM medium: malt extract, 5 g/l; yeast extract, 5 g/l; glucose, 5 g/l.

SMM medium: 8% sodium citrate; 1.5% $(NH_4)_2SO_4$; 0.13% $NaH_2PO_4.2H_2O$; 0.2% $MgSO_4.7H_2O$; 0.1% Tween 80; 0.1% uridine, 0.1% antifoam AF and 7% soya milk. The carbon source (glucose, sucrose, maltose, etc.) is present at a final concentration of 15%. The pH of the medium is adjusted to 6.2 with H_2SO_4.

MDFA medium: 1.2% L-asparagine; 0.8% of salt solution I [2% $Fe(NH_4)_2(SO_4)_2.6H_2O$]; and 14.4% of salt solution II [10.4% K_2HPO_4; 10.2% KH_2PO_4; 1.15% $Na_2CuSO_4.5H_2O$; 0.2%$MgSO_4.7H_2O$; 0.02% $ZnSO_4.7H_2O$; 0.005% $CuSO_4.5H_2O$; 0.05% $CaCl_2.2H_2O$]. The carbon source used was either maltose (usually 6.5%) or a mix of sucrose (3.6%) and glucose (2.7%). Other amounts of carbon source are indicated in each experiment that is described. The initial pH of this medium is 6.5.

C.1.2. Fermentation analysis

Growth and expression studies were conducted in SMM and MDFA media, first in shake flasks, and later in several fermentors equipped with measurement and control systems for the following variables: stirring, dissolved oxygen, pH, antifoam and culture level.

Experiments were conducted in 1-liter shake flasks with a working volume of 150 ml. Inoculation was to a final concentration of 3 x 10^5 spores/ml. Stirring was at 150 rpm, and the incubation temperature was 30°C. The media used was

either SMM or MDFA.

The experiments conducted in the fermentor were analogous to the ones in shake flasks, except that the pH of the medium was maintained constant at a pre-set value, and adjusted by the automatic addition of either 30% NaOH or 0.5N H_2SO_4.

C.1.3. Analytical methods

2-10 ml samples were taken at different times from the fermentation culture and processed to determine the dry weight, thaumatin, maltose and glucose concentrations that were present.

Dry weight was determined by passing a sample through a pre-filter (Nucleopore, Cat.No. 211114). The biological material retained in the pre-filter was washed with 40 ml of pure ethanol and 50 ml of distilled water. It was then incubated at 90°C until a constant weight could be recorded. The filtrate was aliquoted and frozen for further analysis.

Thaumatin concentration in the culture broth was determined by an enzyme-linked immunoassay (ELISA) and by immunoblotting (Western blot) analysis, essentially as described (cf. EP 684312), using an anti-thaumatin polyclonal antibody. For immunoblotting, samples were sometimes concentrated as follows: 500 µl of filtrate were mixed with an equal volume of 10% trichloroacetic acid (TCA), and frozen for 12 h. The sample was then allowed to regain room temperature and centrifuged in a table-top centrifuge (15,000 rpm; 20 min. 4°C). The pellet that is recovered contains all the proteins that were present in this sample. The pellet was then resuspended in protein loading buffer, boiled for 5 minutes, and subjected to SDS-PAGE as described (cf EP 684312).

Approximately 1 ml of filtrate was used for glucose/maltose determination. Glucose levels were determined using a SIGMA

DIAGNOSTICS kit (Procedure number 510).

Maltose concentration in the culture broth was determined as follows: 250 µl of sample filtrate were placed in a test-tube that had been previously chilled; 1.250 ml of anthrone solution (prepared by dissolving 2 g anthrone in 50 ml absolute ethanol and then adding 950 ml of 75% H_2SO_4) were then added, and the sample was kept chilled for five minutes. The sample was then transferred to a boiling water bath, and incubated for 10 minutes. Finally the samples were once again chilled and the absorbance read at 625 nm. Maltose concentrations were determined by comparison to a calibration curve generated by measuring the absorbance of maltose solutions of known concentrations (range: 0 - 0.2 g/l).

C.2. Thaumatin producing strains

C.2.1. Strain TB2b1-44

This strain is a derivative of Lpr66 that was obtained by transformation of the aforementioned LpR66 strain with the expression plasmid pB2KTh-b1. This expression cassette contains the synthetic thaumatin II gene under the control of the promoter of the B2 protein from Acremonium chrysogenum. In shake-flask cultures with MDFA medium this strain secretes 6-8 mg thaumatin/l.

Further optimization studies were performed in a 5-liter New Brunswick fermentor. The inoculum was obtained by growing the strain for 40 hours at 30ºC in CM medium. 450 ml of this inoculum were then used to seed the 5-liter fermentor (working volume of 4.5 liters). RPMs were between 250 and 500, and varied according to the oxygen status of the system, which was always set at 30%.

Different parameters were tested, such as the pH of the medium and the carbon and nitrogen sources. Representative

experiments are described in Figure 12:

1. Growth in MDFA medium with 6.0% sucrose and L-asparagine as the nitrogen source. The set-point for the pH was set at 6.2, and a fed-batch system was installed. Feedings were done at 36, 48, 60 and 72 hours after the beginning of the fermentation. In each feeding, 45 ml of a 0.5 g/ml sucrose solution were added.

2. The conditions were identical to those described under 1 above, but L-asparagine was replaced by ammonium sulfate (the molar amounts were the same in both experiments) as the nitrogen source.

The best productivity was obtained with the conditions described under 1 above, with asparagine as nitrogen source, and with 6% sucrose as the carbon source, with four "feedings" of sucrose every 12 h after 36 h of fermentation. Under these conditions, yields of 100 mg thaumatin/l were obtained.

C.2.2. Strain TGDTh-4

This strain was deposited according to the Budapest Treaty with Access No. CECT20241 on March 25, 1998 (25.03.98) in the following institution:

Colección Española de Cultivos Tipo (CECT)
Edificio de Investigación, planta baja, no. 34
Universidad de Valencia
Campus de Burjasot
46100 Valencia, Spain

It is a derivative of Lpr66 which was obtained by transformation of the aforementioned LpR66 strain with the expression cassette p43GDTh. This expression cassette contains the synthetic thaumatin II gene under the control of

the promoter of the gdhA gene from Aspergillus awamori. In shake-flask cultures with MDFA medium (with 6.0% sucrose) this strain secretes 6-8 mg thaumatin/l.

Experiments were also conducted in the controlled environment of a 5-liter New Brunswick fermentor, as described before for strain TB2b1-44. Ammonium sulfate was used in place of asparagine as nitrogen source, at the same molar levels. In this experiment, also shown in Figure 12, the following conditions were tested: strain TGDTh-4 was grown in MDFA medium supplemented with 6% sucrose and ammonium sulfate as nitrogen source. The pH set-point was 6.2. and a fed-batch system was also installed. Feedings were done at 36, 48, 60 and 72 h after the beginning of the fermentation. In each feeding, 45 ml of a 0.5 g/ml sucrose solution were added.

The results (Fig. 12) indicate that the production of thaumatin is also in the order of 100 mg/l, but with the added advantage of having an earlier production and the use of a more economical nitrogen source. Therefore, it is concluded that the glutamate dehydrogenase promoter from Aspergillus awamori is more efficient than the B2 protein promoter from Acremonium chrysogenum.

C.2.3. Strain TGP-3

This strain is a derivative of Lpr66 which was obtained by transformation of the aforementioned LpR66 strain with the expression cassette pGPThb1. This expression cassette contains the synthetic thaumatin II gene under the control of the promoter of the gpd gene from Aspergillus nidulans. In shake-flask cultures with MDFA medium this strain secretes 9-10 mg thaumatin/liter.

C.2.4. Double transformants

Strains TB2b1-44 and TGP-3 were re-transformed with

expression plasmid pGD71, which contains the thaumatin gene under control of the glutamate dehydrogenase promoter from A. awamori and a hygromycin B resistance gene as a selection marker for transformation experiments. A battery of different transformants (see Table 3) was analyzed in shake flask experiments. It was shown that re-transformation of strain TGP-3 did not result in better producing strains. However, re-transformation of TB2b1-44 did result in better producing strains when cultured in shake-flasks under the standard conditions mentioned before.

Table 3: Production of thaumatin in shake flasks by retransformed strains grown in MDFA medium for 96 h. Quantification by ELISA. All strains were retransformed using hygromicin B resistance as selection marker.

Transformant	Production (mg/l)	Original strain
TGP3-GD1	2.08	TGP3
TGP3-GD2	0.40	TGP3
TGP3-GD3	9.44	TGP3
TGP3-GD4	8.25	TGP3
TGP3-GD5	0.40	TGP3
TGP3-GD6	9.71	TGP3
TB2b1-44-GD1	3.84	TB2b1-44
TB2b1-44-GD2	0.00	TB2b1-44
TB2b1-44-GD3	9.85	TB2b1-44
TB2b1-44-GD4	11.10	TB2b1-44
TB2b1-44-GD5	11.82	TB2b1-44
TB2b1-44-GD6	10.75	TB2b1-44
TB2b1-44-GD7	10.52	TB2b1-44
TB2b1-44-GD8	8.09	TB2b1-44
TB2b1-44-GD9	7.13	TB2b1-44

D: Purification of recombinant thaumatin

Two procedures were employed for the purification of recombinant thaumatin. In the first one the fermentation broth was simply clarified, concentrated and diafiltered, yielding a concentrated and cleaner extract that was used for sensory experiments to ascertain the sweet profile of the recombinant thaumatin. The second procedure involved a classic purification protocol that yielded pure thaumatin.

D.1. Clarification, concentration and diafiltration of the fermentation broth

Biomass was removed by filtration through filter paper. The filtrate was collected in a filtering flask that was submerged in ice. The clarified broth was then centrifugated at 6000 rpm for 15 minutes at 4ºC.

The clarified fermentation broth was further concentrated by ultrafiltration using a ProFluxTM M12 Tangential Filtration System. The system configuration was: base unit, level switch, 2.5 l reservoir, cooling coil, inlet and oulet pressure transducers, secondary pump, one Spiral-wound membrane cartridges S1Y3 (Molecular weight cut-off 3,000 Daltons).

The system was operated as follows: (1) Calibration of the pressure sensors. (2) Adjustment of alarm set points: low inlet pressure 3.0 Bars, high inlet pressure 3.5 Bars, differential pressure 0.3 Bars. (3) Washing of the system and the cartridges with deoinized, distilled water (4) Fill-up of the reservoir with process solution; the solution is kept at 8-10ºC by recirculating cold water (HAAKE, DC1-K20 refrigerated circulator) through the cooling coil. (5) Setting of the level switch at the desired concentration volume (1/4 to 1/5 of the initial volume). (6) Operation of the recirculation pump at 75 %. (7) Adjustment of the Back Pressure Valve to obtain a 3.0 Bar inlet pressure. If

necessary, back pressure was reduced during operation.

Once the fermentation broth was concentrated to the desired volume, the solution was diafiltered in order to remove low molecular weight solutes (Salts, sugars, etc.).

The system configuration allows the operation in the "pumped diafiltration with automatic safety stop" mode. The dialysate (five volumes of deionized water) was transferred by the secondary pump in steps as directed by the level switch. Once the dialysate supply is exhausted, the system and the secondary pump will shut off automatically.

The diafiltered solution is drained from the system, sterilized by filtration (Stericup, 0.22 µm, Millipore) and stored at 4°C.

D.2. Purification of recombinant thaumatin to homogeneity

Recombinant thaumatin was purified to homogeneity using a four step purification scheme that is detailed in Table 4. The starting point for the particular purification protocol that is described here are 500 ml of fermentation broth obtained from the growth of strain TGDTh-4, with thaumatin present at a concentration of 50 mg/l.

Proteins from this broth were precipitated with ammonium sulfate (20-50% range). The precipitate was then re-suspended in 25 mM phosphate buffer, pH 7.0.

This mix was then passed through a Sephadex G-25 column (for desalting purposes) and eluted with the same buffer. Finally the sample was loaded onto a CM-Sepharose column at a flux of 0.5 ml/minute. The column was washed with 25 mM phosphate buffer, pH 7.0 in order to eliminate proteins in the flow-through fraction. Thaumatin was eluted with a NaCl linear gradient (0-400 mM). Thaumatin is eluted from this column in

almost pure form as detected by Coomassie Blue staining.

Table 4: Purification of thaumatin from the fermentation broth for growing strain TGDTh-4 in MDFA medium

SAMPLE	VOLUME (ml)	CONC. (mg/l)	TOTAL (mg)	YIELD (%)
Broth	500	50	25	100
Ammonium sulfate	11	1745	19.2	76.8
Sephadex G-25	30	596	17.9	71.6
CM-Sepharose	24	704	16.9	67.6

While the foregoing illustrative examples are directed to the production of recombinant thaumatin, the production of any other recombinant protein by means of the new methodology provided in the present invention, particularly the new promoter and DNA constructions disclosed herein, is also encompassed by the present invention.

CLAIMS

1. A promoter for the expression of recombinant proteins in filamentous fungi that comprises a nucleotide sequence - or a complementary strand thereof - selected from the group consisting of: (a) the nucleotide sequence numbered 1-740 in the enclosed SEQ ID No. 1; and a nucleotide sequence that hybridizes under stringent conditions to that defined in (a), with the proviso, that the sequence is not the promoter of the gdh gene from Aspergillus nidulans.

2. A promoter according to claim 1 which has the sequence of nucleotides numbered 1-740 in SEQ ID No. 1 or its complementary strand.

3. Isolated promoter of a glutamate dehydrogenase gene from a fungus of the genus Aspergillus with the proviso, that the sequence is not the promoter of the gdh gene from Aspergillus nidulans.

4. Isolated promoter according to claim 3 wherein the fungus is Aspergillus awamori or Aspergillus niger.

5. Isolated promoter according to claim 4 wherein the fungus is Aspergillus awamori.

6. A purified and isolated DNA sequence that encodes a glutamate dehydrogenase protein and that comprises a nucleotide sequence - or a complementary strand thereof - selected from the group consisting of: (a) the nucleotide sequence numbered 741-2245 in the enclosed SEQ ID No. 1; and (b) a nucleotide sequence that hybridizes under stringent conditions to that defined in (a), with the

proviso, that the sequence is not the gdh gene from Aspergillus nidulans.

7. A DNA sequence according to claim 6 which has the sequence of nucleotides numbered 741-2242 in SEQ ID No. 1, or its complementary strand.

8. An isolated DNA sequence encoding a glutamate dehydrogenase from a fungus of the genus Aspergillus, with the proviso, that the sequence is not the gdh gene from Aspergillus nidulans .

9. An isolated DNA sequence according to claim 8 wherein the fungus is Aspergillus awamori or Aspergillus niger.

10. An isolated DNA sequence according to claim 9 wherein the fungus is Aspergillus awamori.

11. The protein encoded by any of the DNA sequences according to claim 6.

12. The protein which has the amino acid sequence in SEQ ID No. 2.

13. An isolated glutamate dehydrogenase from a fungus of the genus Aspergillus with the proviso, that the glutamate dehydrogenase is not the glutamate dehydrogenase from Aspergillus nidulans

14. An isolated glutamate dehydrogenase according to claim 13, wherein the fungus is Aspergillus awamori or Aspergillus niger.

15. An isolated glutamate dehydrogenase according to claim 14, wherein the fungus is Aspergillus awamori.

16. Use of a promoter from a glutamate dehydrogenase gene from a fungus of the genus Aspergillus for the expression of recombinant proteins in filamentous fungi.

17. Use according to claim 16, wherein the promoter is a promoter according to any one of claims 1 to 5.

18. A DNA construction that comprises: a) a promoter from a glutamate dehydrogenase gene from a fungus of the genus Aspergillus; b) a DNA sequence encoding a protein normally expressed from a filamentous fungus or a portion thereof; c) a DNA sequence encoding a cleavable linker peptide; and d) a DNA sequence encoding a desired protein.

19. A DNA construction according to claim 18, wherein the promoter under a) is a promoter according to any one of claims 1 to 5.

20. A DNA construction according to claim 18, wherein the DNA sequence under b) encodes a protein or portion thereof selected from the group consisting of: i) glucoamylase from Aspergillus awamori, Aspergillus niger, Aspergillus oryzae or Aspergillus sojae; ii) B2 from Acremonium chrysogenum; and iii) a glutamate dehydrogenase from a filamentous fungus.

21. A DNA construction according to claim 20, wherein the DNA sequence under b) encodes glucoamylase from Aspergillus awamori Aspergillus niger, Aspergillus oryzae or Aspergillus sojae or a portion thereof.

22. A DNA construction according to claim 20, wherein the DNA sequence under b) encodes the protein B2 from Acremonium chrysogenum or a portion thereof.

23. A DNA construction according to claim 20, wherein the DNA sequence under b) encodes a glutamate dehydrogenase from a filamentous fungus or a portion thereof.

24. A DNA construction according to claim 18, wherein the DNA sequence under c) contains a KEX2 processing sequence.

25. A DNA construction according to any one of claims 18 to 24, wherein the DNA sequence under d) encodes thaumatin.

26. A DNA construction according to claim 25, wherein the DNA sequence under d) is the thaumatin II synthetic gene from plasmid pThIX disclosed in EP 684312.

27. A DNA construction comprising a gdh promoter from a fungus of the genus Aspergillus operatively linked to a DNA sequence encoding a recombinant protein.

28. A DNA construction according to claim 27, wherein the promoter is a promoter according to any one of claims 1 to 5.

29. A filamentous fungus culture capable of producing a recombinant protein which has been transformed with a plasmid containing a DNA construction according to any one of claims 18 to 28.

30. A culture according to claim 29, wherein the filamentous fungus is a fungus from the genus Aspergillus.

31. A culture according to claim 29, wherein the filamentous fungus is selected from the group consisting of Aspergillus awamori, Aspergillus niger, Aspergillus oryzae, Aspergillus nidulans and Aspergillus sojae.

32. A culture according to claim 29, wherein the plasmid contains a DNA construction according to any one of claims 25 or 26.

33. A process for producing a recombinant protein in a filamentous fungus comprising the following steps:
a) preparation of an expression plasmid containing a DNA construct according to any of claims 18 to 28;
b) transformation of a strain of filamentous fungus with said expression plasmid;
c) culture of the transformed strain under appropriate nutrient conditions to produce the desired protein, either intracellularly, extracellularly or in both ways simultaneosly; and
d) depending on the case, separation and purification of the desired protein from the fermentation broth.

34. A process according to claim 33, wherein the recombinant protein is thaumatin and the expression plasmid contains a DNA construction according to claims 25 or 26.

35. Use of a DNA sequence derived from a nucleotide sequence according to claims 1 to 6 as a probe for the identification and isolation of a glutamate dehydrogenase gene and/or a promoter sequence of a glutamate dehydrogenase gene.

SEQUENCE LISTING

SEQ ID 1. Nucleotide sequence of 2570 bp of a DNA fragment present in plasmid pBSGh, as well as the 5′ end from plasmid pB1.7, which contains the gdhA gene of A. awamori. The numbers to the right indicate the numbering of the sequence. The promoter region of the gdhA gene of A. awamori precedes the initiating ATG codon at position 741 in the sequence. The initiation of transcription is at T of position -86 with respect to the translation initiation triplet (position 655 in the sequence). The part of the gene encoding the protein begins at position 741. The numbers underneath the amino acids refer to their numbering. There are a total of 460 amino acids. The two introns present are also shown.

```
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CCCGAACTTC GGCGGCCACC GCCAAACCCG CCCCGGAAGC CGGCCTCATT TGCCGTTTGG     360
GCGTGCCAGG AAATGCCGCC TGCAGCGGAG ACTCCCTAGT GTGGTCTGTG TTGCCTGTGT     420
CGTCTGTGTA GTATACTAGT TACTAGTCTA CTACTGTACA GTGGATGGCC TGAGGGGGGG     480
ACTTTATGTC CGACTCCGGC TGTTCTCCTC CCTCTATCCA CTCTACCCTC TTCCCTCTCT     540
TCTGTCTTTC TCCCCGCTCT CGCCCCTCCC CTCCTCGAAA ACATAAATCG GCCTTTCCCC     600
CTCGCCATCT TCTTCTTCTT CTCCCTCTCC TTTCTCTTTC TTCTTCAGAC TACTTCTCTT     660
TCTTTCATCT TTTCTCTATA TTCCTGTTTT CCTAGATACC CCAGTTAAAA AAGTTCTCTC     720
AATCAATCCT CCCCTTCAGA ATG TCT AAC CTT CCT CAC GAG CCC GAG TTC          770
                      Met Ser Asn Leu Pro His Glu Pro Glu Phe
                      1               5                  10
GAG CAG GCC TAC AAG GGTATGTTCC ATTGCCCCTC CGAAATTGAT GATGGAAAAA      825
Glu Gln Ala Tyr Lys
            15
AAATTCTAAC AACATCCTCT TACA GAG CTT GCC TCG ACC CTT GAG AAC TCC        876
                           Glu Leu Ala Ser Thr Leu Glu Asn Ser
                                       20
ACC CTC TTC CAG AAG AAC CCC GAA TAC CGC AAG GCC CTT GCT GTC GTC      924
Thr Leu Phe Gln Lys Asn Pro Glu Tyr Arg Lys Ala Leu Ala Val Val
25                  30                  35                  40
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TCC GTC CCC GAG CGT GTC ATC CAG TTC CGT GTC GTC TGG GAG GAT GAT      972
Ser Val Pro Glu Arg Val Ile Gln Phe Arg Val Val Trp Glu Asp Asp
                  45                  50                  55

GCC GGC AAC GTC CAG GTC AAC CGC GGT TTC CGT GTC CAG TTC AAC AGC     1020
Ala Gly Asn Val Gln Val Asn Arg Gly Phe Arg Val Gln Phe Asn Ser
              60                  65                  70

GCC CTC GGT CCC TAC AAG GGT GGT CTT CGT TTC CAC CCC TCC GTC AAC     1068
Ala Leu Gly Pro Tyr Lys Gly Gly Leu Arg Phe His Pro Ser Val Asn
          75                  80                  85

TTG TCC ATC CTC AAG TTC CTT GGT TTC GAG CAG ATC TTC AAG AAT GCT     1116
Leu Ser Ile Leu Lys Phe Leu Gly Phe Glu Gln Ile Phe Lys Asn Ala
      90                  95                  100

CTC ACT GGC CTG AAC ATG GGT GGT GGT AAG GGT GGT TCC GAC TTC GAC     1164
Leu Thr Gly Leu Asn Met Gly Gly Gly Lys Gly Gly Ser Asp Phe Asp
105                 110                 115                 120

CCC AAG GGC AAG TCC GAC AAC GAG ATC CGT CGC TTC TGT GTT TCC TTC     1212
Pro Lys Gly Lys Ser Asp Asn Glu Ile Arg Arg Phe Cys Val Ser Phe
                  125                 130                 135

ATG ACC GAG CTC TGC AAG CAC ATC GGT GCC GAC ACT GAT GTT CCC GCT     1260
Met Thr Glu Leu Cys Lys His Ile Gly Ala Asp Thr Asp Val Pro Ala
              140                 145                 150

GGT GAC ATC GGT GTC ACC GGT CGT GAG GTC GGT TTC CTC TTC GGC CAG     1308
Gly Asp Ile Gly Val Thr Gly Arg Glu Val Gly Phe Leu Phe Gly Gln
          155                 160                 165

TAC CGC AAG ATC CGC AAC CAG TGG GAG GGT GTT CTC ACC GGT AAG GGT     1356
Tyr Arg Lys Ile Arg Asn Gln Trp Glu Gly Val Leu Thr Gly Lys Gly
      170                 175                 180

GGC AGC TGG GGT GGT TCC CTC ATC CGC CCT GAG GCC ACC GGT TAC GGT     1404
Gly Ser Trp Gly Gly Ser Leu Ile Arg Pro Glu Ala Thr Gly Tyr Gly
185                 190                 195                 200

GTT GTC TAC GTATGTCAAT TCCTCTTCTT ATGATTATCT ATGTATAACA             1453
Val Val Tyr

GCGACTAACG CGTAACAG TAC GTC GAG CAC ATG ATT GCT CAC GCC ACC AAC     1504
                      Tyr Val Glu His Met Ile Ala His Ala Thr Asn
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Gly Gln Glu Ser Phe Lys Gly Lys Arg Val Ala Ile Ser Gly Ser Gly
215                 220                 225                 230

AAC GTT GCC CAG TAC GCC GCC CTC AAG GTC ATT GAG CTC GGC GGT TCC     1600
Asn Val Ala Gln Tyr Ala Ala Leu Lys Val Ile Glu Leu Gly Gly Ser
                  235                 240                 245

GTC GTC TCC CTG AGC GAC ACG CAG GGC TCC CTC ATC ATC AAC GGC GAG     1648
Val Val Ser Leu Ser Asp Thr Gln Gly Ser Leu Ile Ile Asn Gly Glu
              250                 255                 260

GGT AGC TTC ACC CCC GAG GAG ATC GAG CTC ATC GCT CAG ACC AAG GTC     1696
Gly Ser Phe Thr Pro Glu Glu Ile Glu Leu Ile Ala Gln Thr Lys Val
          265                 270                 275

GAG CGC AAC GAG CTC GCC AGC ATC GTC GGT GCT GCT CCC TTC AGC GAC     1744
Glu Arg Asn Glu Leu Ala Ser Ile Val Gly Ala Ala Pro Phe Ser Asp
      280                 285                 290

GCC AAC AAG TTC AAG TAC ATT GCT GGT GCC CGC CCC TGG GTT CAC GTC     1792
Ala Asn Lys Phe Lys Tyr Ile Ala Gly Ala Arg Pro Trp Val His Val
295                 300                 305                 310
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Gly Lys Val Asp Val Ala Leu Pro Ser Ala Thr Gln Asn Glu Val Ser
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Gly Glu Glu Ala Gln Val Leu Ile Asn Ala Gly Cys Lys Phe Ile Ala
            330                 335                 340

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Glu Gly Ser Asn Met Gly Cys Thr Gln Glu Ala Ile Asp Thr Phe Glu
        345                 350                 355

GCC CAC CGT ACC GCC AAC GCT GGC GCG GCT GCC ATC TGG TAC GCC CCC      1984
Ala His Arg Thr Ala Asn Ala Gly Ala Ala Ala Ile Trp Tyr Ala Pro
    360                 365                 370

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Gly Lys Ala Ala Asn Ala Gly Gly Val Ala Val Ser Gly Leu Glu Met
375                 380                 385                 390

GCT CAG AAC TCT GCC CGC CTC AGC TGG ACT TCT GAG GAG GTT GAT GCC      2080
Ala Gln Asn Ser Ala Arg Leu Ser Trp Thr Ser Glu Glu Val Asp Ala
                395                 400                 405

CGT CTT AAG GAC ATC ATG CGC GAC TGC TTC AAG AAC GGT CTT GAG ACT      2128
Arg Leu Lys Asp Ile Met Arg Asp Cys Phe Lys Asn Gly Leu Glu Thr
            410                 415                 420

GCT CAG GAG TAC GCC ACC CCC GCT GAG GGT GTC CTG CCT TCC CTG GTG      2176
Ala Gln Glu Tyr Ala Thr Pro Ala Glu Gly Val Leu Pro Ser Leu Val
        425                 430                 435

ACC GGA TCC AAC ATT GCC GGT TTC ACC AAG GTG GCT GCC GCC ATG AAG      2224
Thr Gly Ser Asn Ile Ala Gly Phe Thr Lys Val Ala Ala Ala Met Lys
    440                 445                 450

GAC CAG GGT GAC TGG TGG TAAATGCGGA AAGCCGCAAA CCCCCGCGGC             2272
Asp Gln Gly Asp Trp Trp
455                 460

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CTTGTAATTT GTTGTATCAA TAGTAGATAA TACAACTGTA GTCAACTACC CTTGCATCTT    2452

CACTATTTGC AGATGCATTC ATCTCTATTC CGAGCACATG CACAAACCCA TGGGACCGCA    2512

GTTCACTAGT ACTTAGCCTG TTATCTTCCC TCTATCGCAT CTTAAACAAC TATCTAGA      2570
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SEQ ID 2. Amino acid sequence of the glutamate dehydrogenase A (gdh A) protein from Aspergillus awamori as deduced from the nucleotide sequence in SEQ ID 1.

```
Met Ser Asn Leu Pro His Glu Pro Glu Phe Glu Gln Ala Tyr Lys Glu
 1               5                  10                  15

Leu Ala Ser Thr Leu Glu Asn Ser Thr Leu Phe Gln Lys Asn Pro Glu
            20                  25                  30

Tyr Arg Lys Ala Leu Ala Val Val Ser Val Pro Glu Arg Val Ile Gln
        35                  40                  45
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Phe Arg Val Val Trp Glu Asp Asp Ala Gly Asn Val Gln Val Asn Arg
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Gly Phe Arg Val Gln Phe Asn Ser Ala Leu Gly Pro Tyr Lys Gly Gly
65                   70                  75                  80

Leu Arg Phe His Pro Ser Val Asn Leu Ser Ile Leu Lys Phe Leu Gly
                85                  90                  95

Phe Glu Gln Ile Phe Lys Asn Ala Leu Thr Gly Leu Asn Met Gly Gly
            100                 105                 110

Gly Lys Gly Gly Ser Asp Phe Asp Pro Lys Gly Lys Ser Asp Asn Glu
        115                 120                 125

Ile Arg Arg Phe Cys Val Ser Phe Met Thr Glu Leu Cys Lys His Ile
    130                 135                 140

Gly Ala Asp Thr Asp Val Pro Ala Gly Asp Ile Gly Val Thr Gly Arg
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Glu Val Gly Phe Leu Phe Gly Gln Tyr Arg Lys Ile Arg Asn Gln Trp
                165                 170                 175

Glu Gly Val Leu Thr Gly Lys Gly Gly Ser Trp Gly Gly Ser Leu Ile
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Arg Pro Glu Ala Thr Gly Tyr Gly Val Val Tyr Tyr Val Glu His Met
        195                 200                 205

Ile Ala His Ala Thr Asn Gly Gln Glu Ser Phe Lys Gly Lys Arg Val
    210                 215                 220

Ala Ile Ser Gly Ser Gly Asn Val Ala Gln Tyr Ala Ala Leu Lys Val
225                 230                 235                 240

Ile Glu Leu Gly Gly Ser Val Val Ser Leu Ser Asp Thr Gln Gly Ser
                245                 250                 255

Leu Ile Ile Asn Gly Glu Gly Ser Phe Thr Pro Glu Glu Ile Glu Leu
            260                 265                 270

Ile Ala Gln Thr Lys Val Glu Arg Asn Glu Leu Ala Ser Ile Val Gly
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    290                 295                 300

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Thr Gln Asn Glu Val Ser Gly Glu Glu Ala Gln Val Leu Ile Asn Ala
                325                 330                 335

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Ala Ile Asp Thr Phe Glu Ala His Arg Thr Ala Asn Ala Gly Ala Ala
        355                 360                 365
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Ser Glu Glu Val Asp Ala Arg Leu Lys Asp Ile Met Arg Asp Cys Phe
                405                 410                 415

Lys Asn Gly Leu Glu Thr Ala Gln Glu Tyr Ala Thr Pro Ala Glu Gly
            420                 425                 430

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SEQUENCE LISTING

<110> URQUIMA, S.A.

<120> Promotor and constructions for expression of recombinant proteins in filamentous fungi

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gcgtgccagg aaatgccgcc tgcagcggag actccctagt gtggtctgtg ttgcctgtgt 420

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ctcgccatct tcttcttctt ctcccctctcc tttctctttc ttcttcagac tacttctctt 660

tctttcatct tttctctata ttcctgtttt cctagatacc ccagttaaaa aagttctctc 720

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cag gcc tac aag ggtatgttcc attgcccctc cgaaattgat gatggaaaaa       825
Gln Ala Tyr Lys
            15

aaattctaac aacatcctct taca gag ctt gcc tcg acc ctt gag aac tcc    876
                           Glu Leu Ala Ser Thr Leu Glu Asn Ser
                                           20

acc ctc ttc cag aag aac ccc gaa tac cgc aag gcc ctt gct gtc gtc   924
Thr Leu Phe Gln Lys Asn Pro Glu Tyr Arg Lys Ala Leu Ala Val Val
 25                  30                  35                  40

tcc gtc ccc gag cgt gtc atc cag ttc cgt gtc gtc tgg gag gat gat   972
Ser Val Pro Glu Arg Val Ile Gln Phe Arg Val Val Trp Glu Asp Asp
                 45                  50                  55

gcc ggc aac gtc cag gtc aac cgc ggt ttc cgt gtc cag ttc aac agc   1020
Ala Gly Asn Val Gln Val Asn Arg Gly Phe Arg Val Gln Phe Asn Ser
             60                  65                  70

gcc ctc ggt ccc tac aag ggt ggt ctt cgt ttc cac ccc tcc gtc aac   1068
Ala Leu Gly Pro Tyr Lys Gly Gly Leu Arg Phe His Pro Ser Val Asn
         75                  80                  85

ttg tcc atc ctc aag ttc ctt ggt ttc gag cag atc ttc aag aat gct   1116
Leu Ser Ile Leu Lys Phe Leu Gly Phe Glu Gln Ile Phe Lys Asn Ala
     90                  95                  100

ctc act ggc ctg aac atg ggt ggt ggt aag ggt ggt tcc gac ttc gac   1164
Leu Thr Gly Leu Asn Met Gly Gly Gly Lys Gly Gly Ser Asp Phe Asp
105                 110                 115                 120

ccc aag ggc aag tcc gac aac gag atc cgt cgc ttc tgt gtt tcc ttc   1212
Pro Lys Gly Lys Ser Asp Asn Glu Ile Arg Arg Phe Cys Val Ser Phe
                125                 130                 135

atg acc gag ctc tgc aag cac atc ggt gcc gac act gat gtt ccc gct   1260
Met Thr Glu Leu Cys Lys His Ile Gly Ala Asp Thr Asp Val Pro Ala
            140                 145                 150

ggt gac atc ggt gtc acc ggt cgt gag gtc ggt ttc ctc ttc ggc cag   1308
Gly Asp Ile Gly Val Thr Gly Arg Glu Val Gly Phe Leu Phe Gly Gln
        155                 160                 165

tac cgc aag atc cgc aac cag tgg gag ggt gtt ctc acc ggt aag ggt   1356
Tyr Arg Lys Ile Arg Asn Gln Trp Glu Gly Val Leu Thr Gly Lys Gly
    170                 175                 180
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ggc agc tgg ggt ggt tcc ctc atc cgc cct gag gcc acc ggt tac ggt   1404
Gly Ser Trp Gly Gly Ser Leu Ile Arg Pro Glu Ala Thr Gly Tyr Gly
185                 190                 195                 200

gtt gtc tac gtatgtcaat tcctcttctt atgattatct atgtataaca           1453
Val Val Tyr

gcgactaacg cgtaacag tac gtc gag cac atg att gct cac gcc acc aac   1504
                    Tyr Val Glu His Met Ile Ala His Ala Thr Asn
                        205                 210

ggc cag gag tcc ttc aag ggc aag cgc gtt gcc atc tcc ggt tcc ggt   1552
Gly Gln Glu Ser Phe Lys Gly Lys Arg Val Ala Ile Ser Gly Ser Gly
215                 220                 225                 230

aac gtt gcc cag tac gcc gcc ctc aag gtc att gag ctc ggc ggt tcc   1600
Asn Val Ala Gln Tyr Ala Ala Leu Lys Val Ile Glu Leu Gly Gly Ser
                235                 240                 245

gtc gtc tcc ctg agc gac acg cag ggc tcc ctc atc atc aac ggc gag   1648
Val Val Ser Leu Ser Asp Thr Gln Gly Ser Leu Ile Ile Asn Gly Glu
            250                 255                 260

ggt agc ttc acc ccc gag gag atc gag ctc atc gct cag acc aag gtc   1696
Gly Ser Phe Thr Pro Glu Glu Ile Glu Leu Ile Ala Gln Thr Lys Val
        265                 270                 275

gag cgc aac gag ctc gcc agc atc gtc ggt gct gct ccc ttc agc gac   1744
Glu Arg Asn Glu Leu Ala Ser Ile Val Gly Ala Ala Pro Phe Ser Asp
    280                 285                 290

gcc aac aag ttc aag tac att gct ggt gcc cgc ccc tgg gtt cac gtc   1792
Ala Asn Lys Phe Lys Tyr Ile Ala Gly Ala Arg Pro Trp Val His Val
295                 300                 305                 310

ggc aag gtc gac gtc gct ctc ccc tcc gct acc cag aac gaa gtt tcc   1840
Gly Lys Val Asp Val Ala Leu Pro Ser Ala Thr Gln Asn Glu Val Ser
                315                 320                 325

ggc gag gag gcc cag gtc ctc atc aac gct ggc tgc aag ttc atc gcc   1888
Gly Glu Glu Ala Gln Val Leu Ile Asn Ala Gly Cys Lys Phe Ile Ala
            330                 335                 340

gag ggt tcc aac atg ggt tgc acc cag gag gcc atc gac acc ttc gag   1936
Glu Gly Ser Asn Met Gly Cys Thr Gln Glu Ala Ile Asp Thr Phe Glu
        345                 350                 355

gcc cac cgt acc gcc aac gct ggc gcg gct gcc atc tgg tac gcc ccc   1984
Ala His Arg Thr Ala Asn Ala Gly Ala Ala Ala Ile Trp Tyr Ala Pro
    360                 365                 370

ggt aag gcc gcc aac gcc ggt ggt gtc gct gtc tcc ggt ctg gag atg   2032
Gly Lys Ala Ala Asn Ala Gly Gly Val Ala Val Ser Gly Leu Glu Met
375                 380                 385                 390

gct cag aac tct gcc cgc ctc agc tgg act tct gag gag gtt gat gcc   2080
Ala Gln Asn Ser Ala Arg Leu Ser Trp Thr Ser Glu Glu Val Asp Ala
                395                 400                 405
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cgt ctt aag gac atc atg cgc gac tgc ttc aag aac ggt ctt gag act   2128
Arg Leu Lys Asp Ile Met Arg Asp Cys Phe Lys Asn Gly Leu Glu Thr
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gct cag gag tac gcc acc ccc gct gag ggt gtc ctg cct tcc ctg gtg   2176
Ala Gln Glu Tyr Ala Thr Pro Ala Glu Gly Val Leu Pro Ser Leu Val
        425                 430                 435

acc gga tcc aac att gcc ggt ttc acc aag gtg gct gcc gcc atg aag   2224
Thr Gly Ser Asn Ile Ala Gly Phe Thr Lys Val Ala Ala Ala Met Lys
    440                 445                 450

gac cag ggt gac tgg tgg taaatgcgga aagccgcaaa ccccccgcggc          2272
Asp Gln Gly Asp Trp Trp
455                 460

ttatgtcatg acgattatgt agtttgatgt tccctttcag cgcggatgga tagaggcgcc 2332

ggtgtttttct tgctagttta gatggatgca taatgatatc cttttcttaa tcctcaaatt 2392

cttgtaattt gttgtatcaa tagtagataa tacaactgta gtcaactacc cttgcatctt 2452

cactatttgc agatgcattc atctctattc cgagcacatg cacaaaccca tgggaccgca 2512

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<213> Aspergillus awamorii

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Leu Ala Ser Thr Leu Glu Asn Ser Thr Leu Phe Gln Lys Asn Pro Glu
             20                  25                  30

Tyr Arg Lys Ala Leu Ala Val Val Ser Val Pro Glu Arg Val Ile Gln
         35                  40                  45

Phe Arg Val Val Trp Glu Asp Asp Ala Gly Asn Val Gln Val Asn Arg
     50                  55                  60

Gly Phe Arg Val Gln Phe Asn Ser Ala Leu Gly Pro Tyr Lys Gly Gly
 65                  70                  75                  80

Leu Arg Phe His Pro Ser Val Asn Leu Ser Ile Leu Lys Phe Leu Gly
                 85                  90                  95

Phe Glu Gln Ile Phe Lys Asn Ala Leu Thr Gly Leu Asn Met Gly Gly
            100                 105                 110

Gly Lys Gly Gly Ser Asp Phe Asp Pro Lys Gly Lys Ser Asp Asn Glu
        115                 120                 125

Ile Arg Arg Phe Cys Val Ser Phe Met Thr Glu Leu Cys Lys His Ile
    130                 135                 140
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Gly Ala Asp Thr Asp Val Pro Ala Gly Asp Ile Gly Val Thr Gly Arg
145                 150                 155                 160

Glu Val Gly Phe Leu Phe Gly Gln Tyr Arg Lys Ile Arg Asn Gln Trp
                165                 170                 175

Glu Gly Val Leu Thr Gly Lys Gly Gly Ser Trp Gly Gly Ser Leu Ile
            180                 185                 190

Arg Pro Glu Ala Thr Gly Tyr Gly Val Val Tyr Tyr Val Glu His Met
        195                 200                 205

Ile Ala His Ala Thr Asn Gly Gln Glu Ser Phe Lys Gly Lys Arg Val
    210                 215                 220

Ala Ile Ser Gly Ser Gly Asn Val Ala Gln Tyr Ala Ala Leu Lys Val
225                 230                 235                 240

Ile Glu Leu Gly Gly Ser Val Val Ser Leu Ser Asp Thr Gln Gly Ser
                245                 250                 255

Leu Ile Ile Asn Gly Glu Gly Ser Phe Thr Pro Glu Glu Ile Glu Leu
            260                 265                 270

Ile Ala Gln Thr Lys Val Glu Arg Asn Glu Leu Ala Ser Ile Val Gly
        275                 280                 285

Ala Ala Pro Phe Ser Asp Ala Asn Lys Phe Lys Tyr Ile Ala Gly Ala
    290                 295                 300

Arg Pro Trp Val His Val Gly Lys Val Asp Val Ala Leu Pro Ser Ala
305                 310                 315                 320

Thr Gln Asn Glu Val Ser Gly Glu Glu Ala Gln Val Leu Ile Asn Ala
                325                 330                 335

Gly Cys Lys Phe Ile Ala Glu Gly Ser Asn Met Gly Cys Thr Gln Glu
            340                 345                 350

Ala Ile Asp Thr Phe Glu Ala His Arg Thr Ala Asn Ala Gly Ala Ala
        355                 360                 365

Ala Ile Trp Tyr Ala Pro Gly Lys Ala Ala Asn Ala Gly Gly Val Ala
    370                 375                 380

Val Ser Gly Leu Glu Met Ala Gln Asn Ser Ala Arg Leu Ser Trp Thr
385                 390                 395                 400

Ser Glu Glu Val Asp Ala Arg Leu Lys Asp Ile Met Arg Asp Cys Phe
                405                 410                 415

Lys Asn Gly Leu Glu Thr Ala Gln Glu Tyr Ala Thr Pro Ala Glu Gly
            420                 425                 430

Val Leu Pro Ser Leu Val Thr Gly Ser Asn Ile Ala Gly Phe Thr Lys
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Val Ala Ala Ala Met Lys Asp Gln Gly Asp Trp Trp
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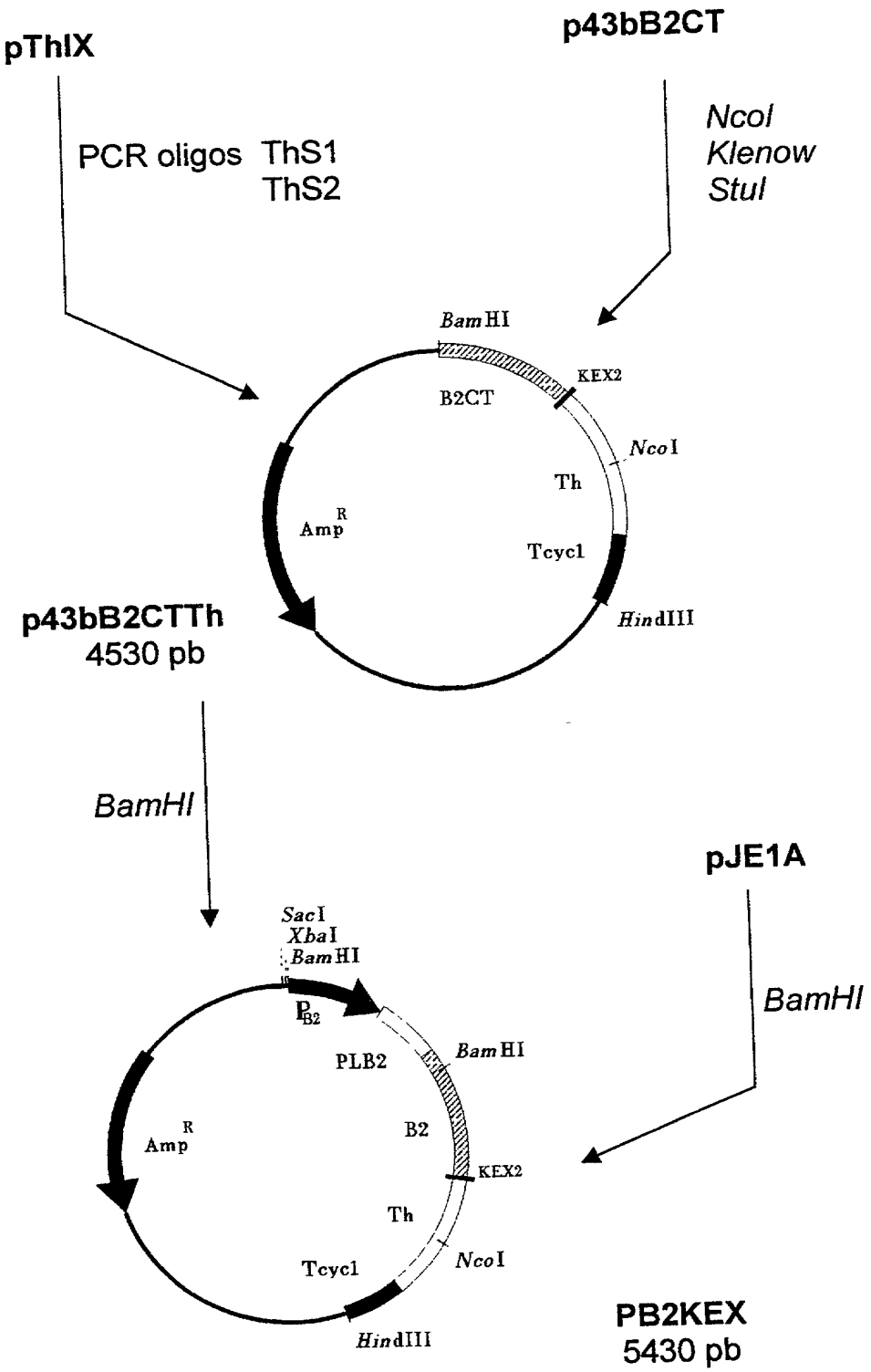
Fig. 1A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PCR oligos ThS1
ThS2
NcoI
Klenow
StuI
BamHI
KEX2
B2CT
NcoI
Th
AmpR
Tcycl
HindIII
p43bB2CTTh
4530 pb
BamHI
pJE1A
SacI
XbaI
BamHI
PB2
PLB2
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NcoI
HindIII
BamHI
PB2KEX
5430 pb

Fig. 1B

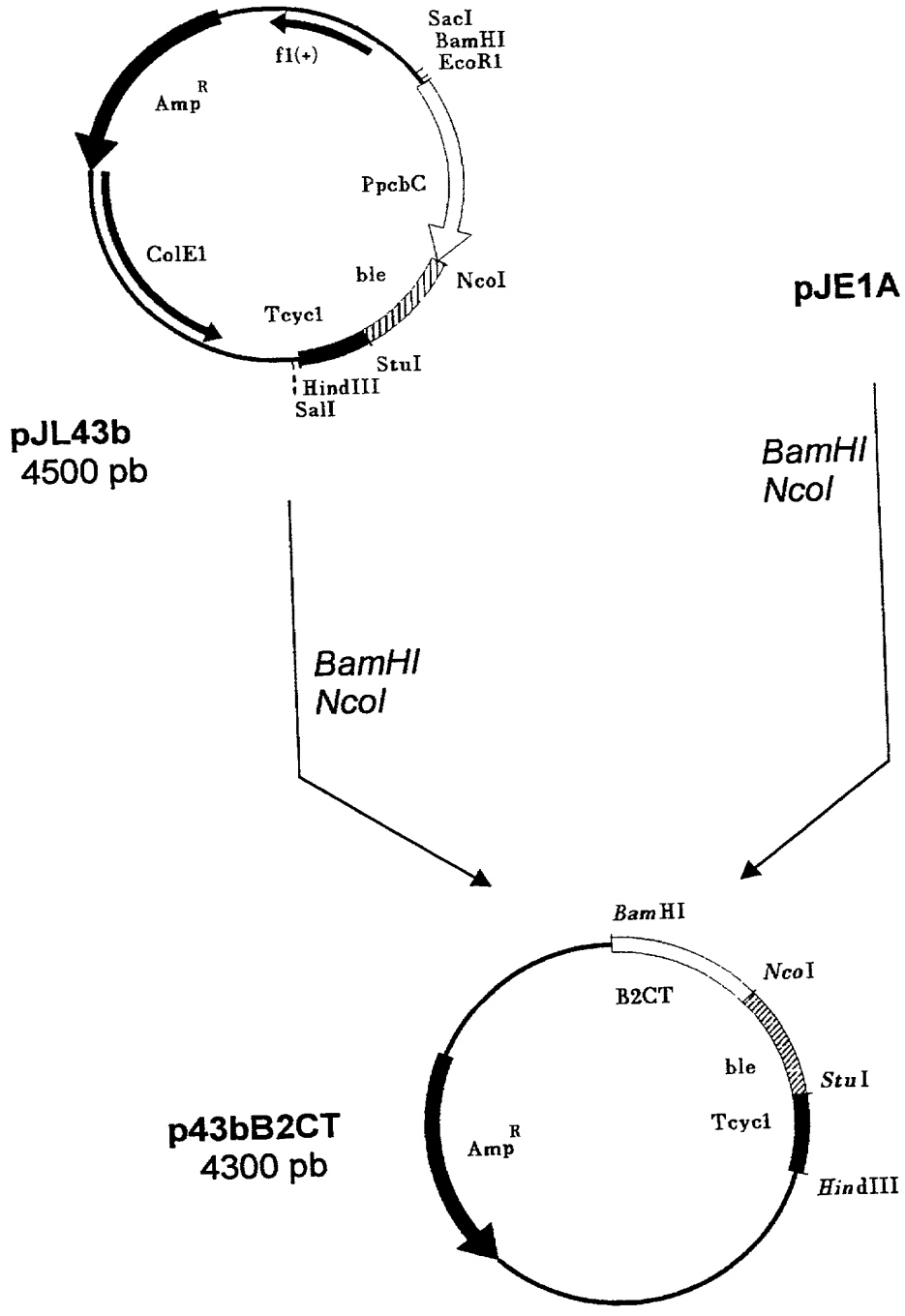
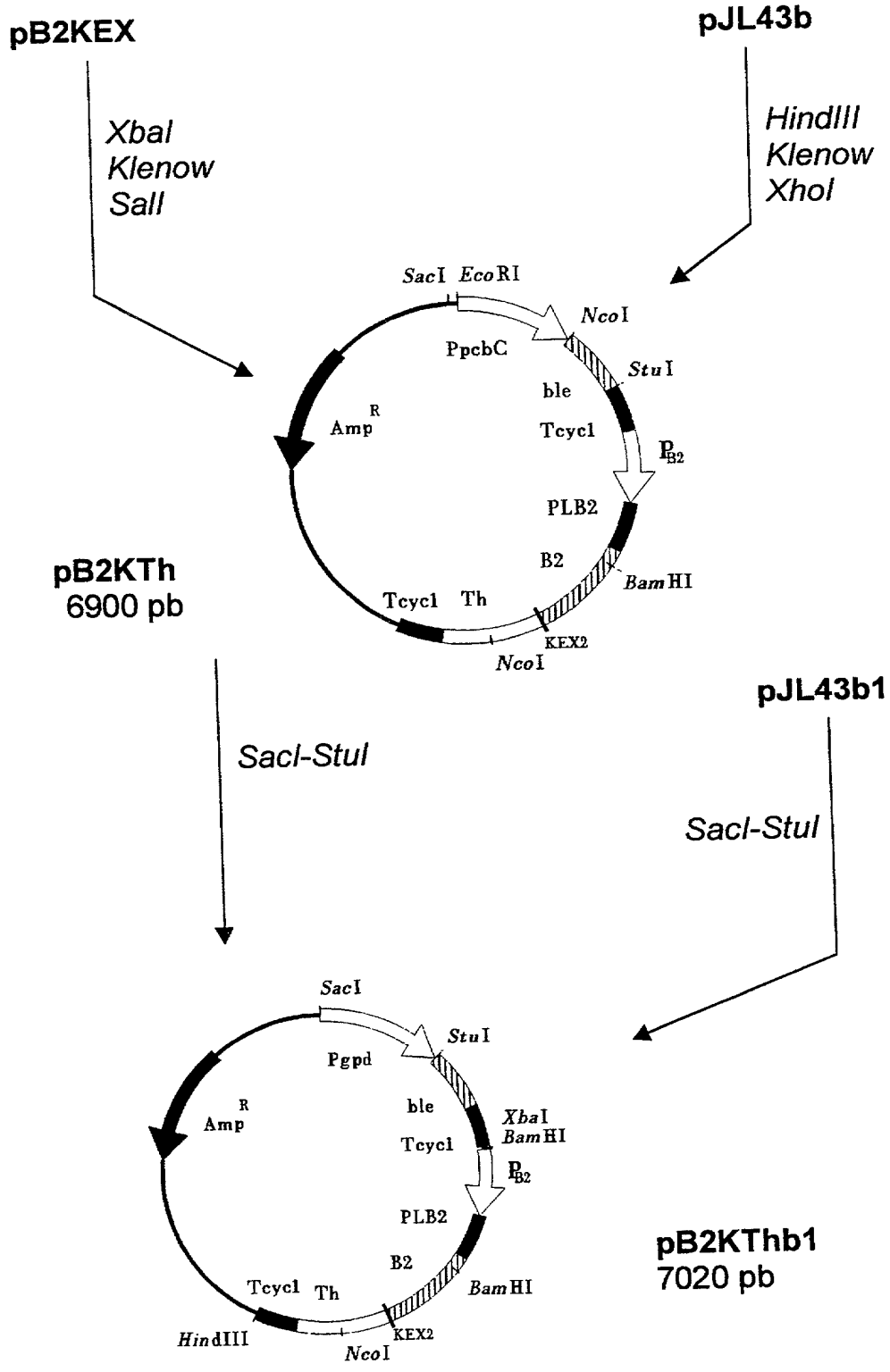

SacI
BamHI
EcoR1
f1(+)
Amp R
PpcbC
ColE1
ble
NcoI
Tcyc1
StuI
HindIII
SalI
pJL43b
4500 pb
pJE1A
BamHI
NcoI
BamHI
NcoI
BamHI
NcoI
B2CT
ble
StuI
Tcyc1
Amp R
HindIII
p43bB2CT
4300 pb

Fig. 1C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pJL43b
XbaI
Klenow
SalI
HindIII
Klenow
XhoI
SacI EcoRI
NcoI
PpcbC
StuI
ble
Amp R
Tcyc1
PB2
PLB2
B2
BamHI
Tcyc1 Th
KEX2
NcoI
pB2KTh
6900 pb
SacI-StuI
pJL43b1
SacI-StuI
SacI
StuI
Pgpd
ble
Amp R
XbaI
BamHI
Tcyc1
PB2
PLB2
B2
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BamHI
HindIII
KEX2
NcoI
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7020 pb

Fig. 2

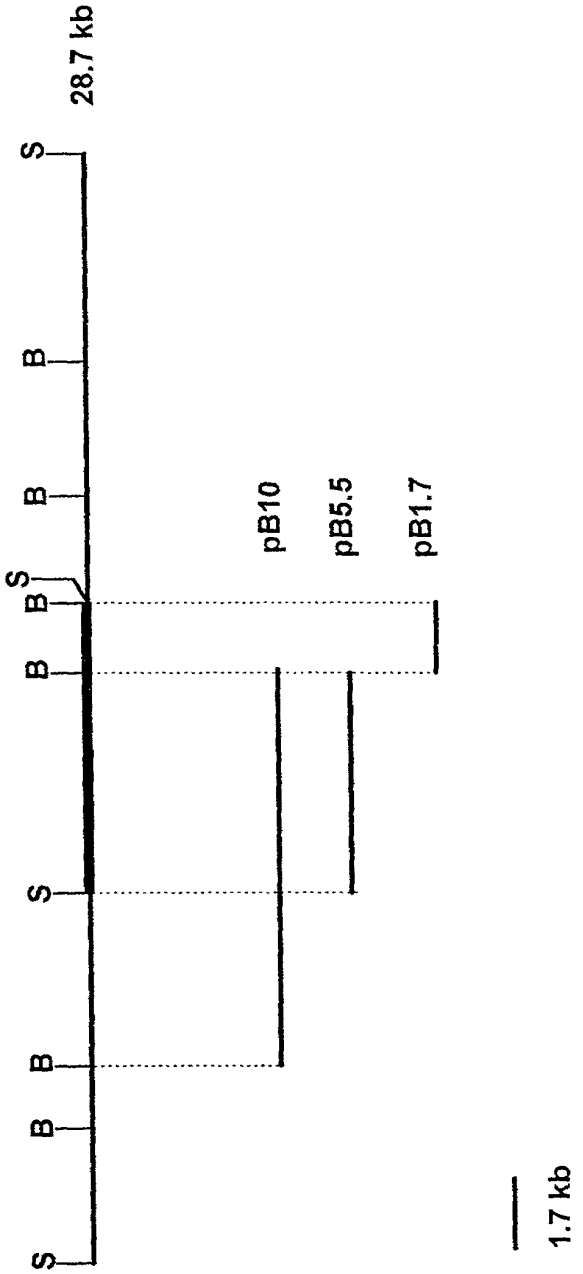


Fig. 3

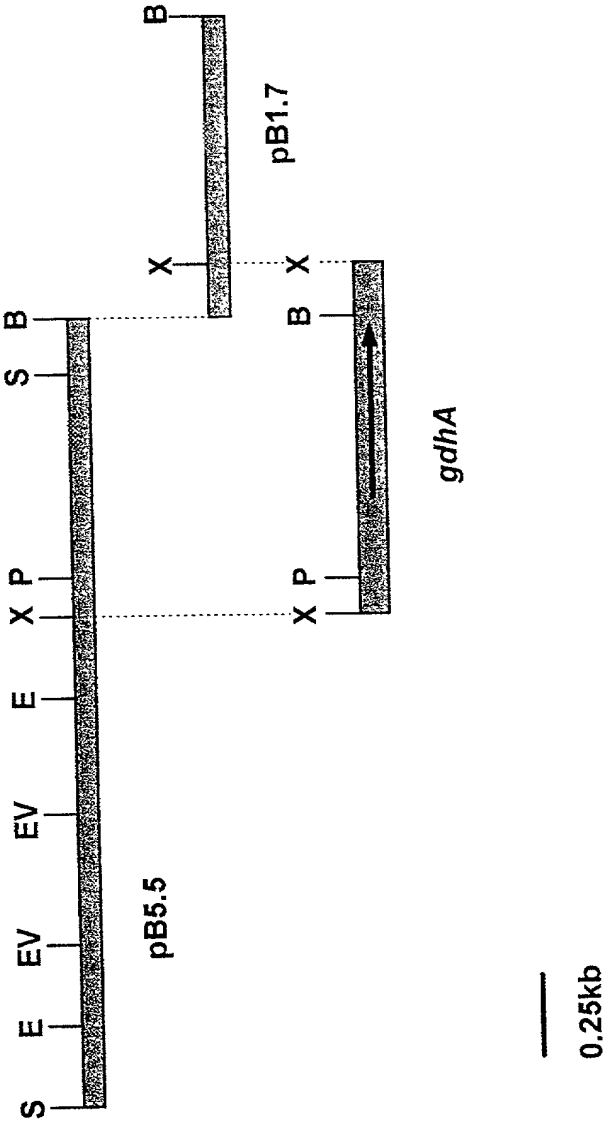


Fig. 4A

Fig. 4B

Fig. 5

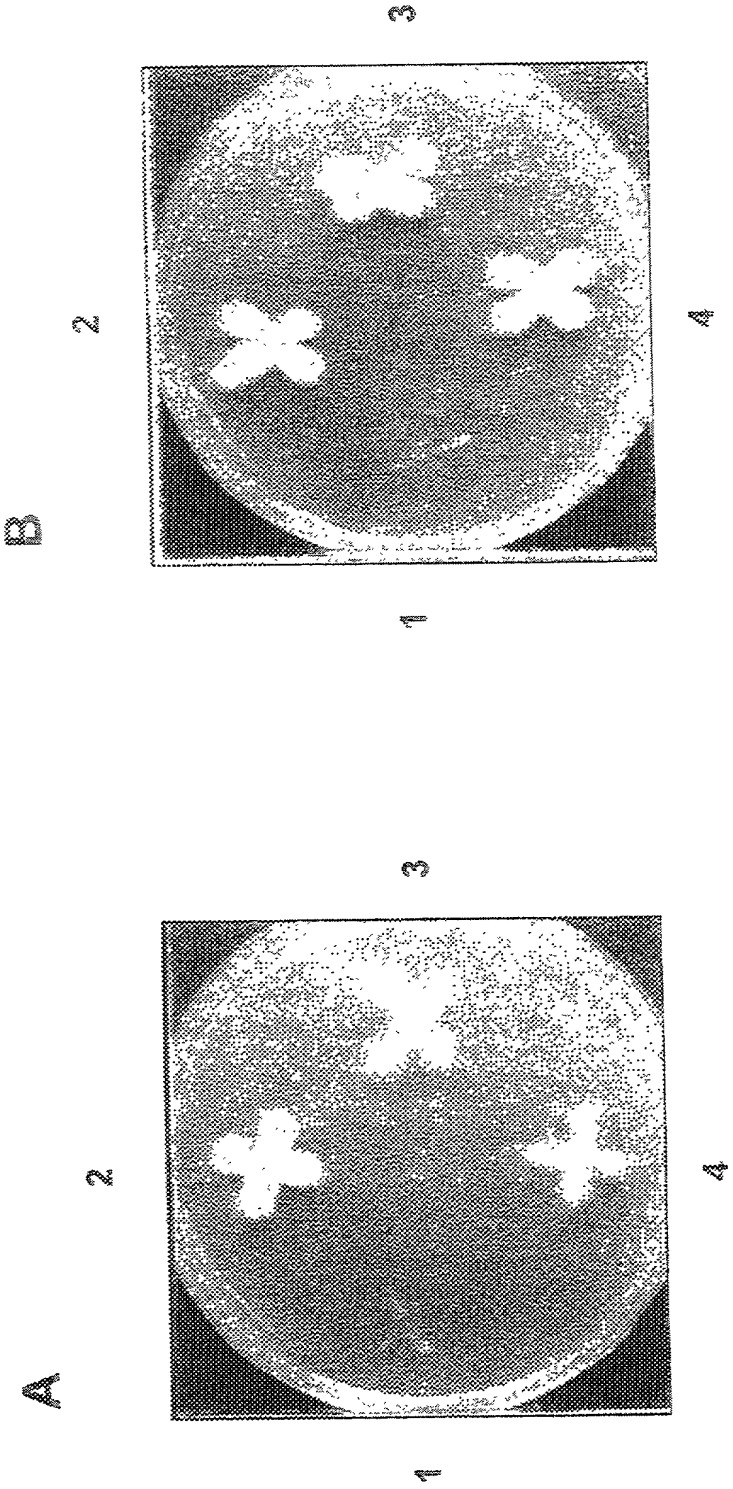


Fig. 6

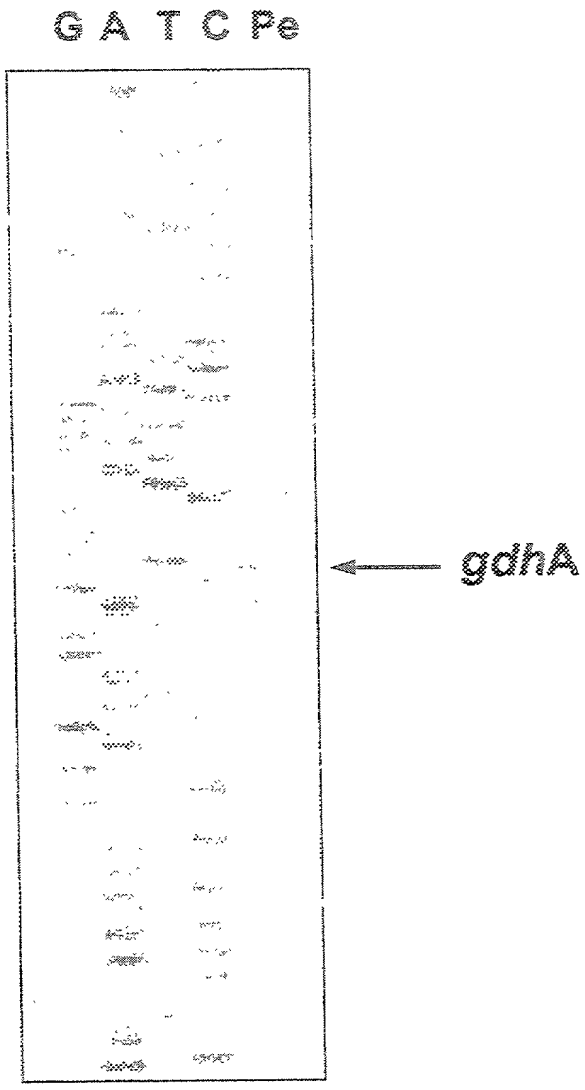
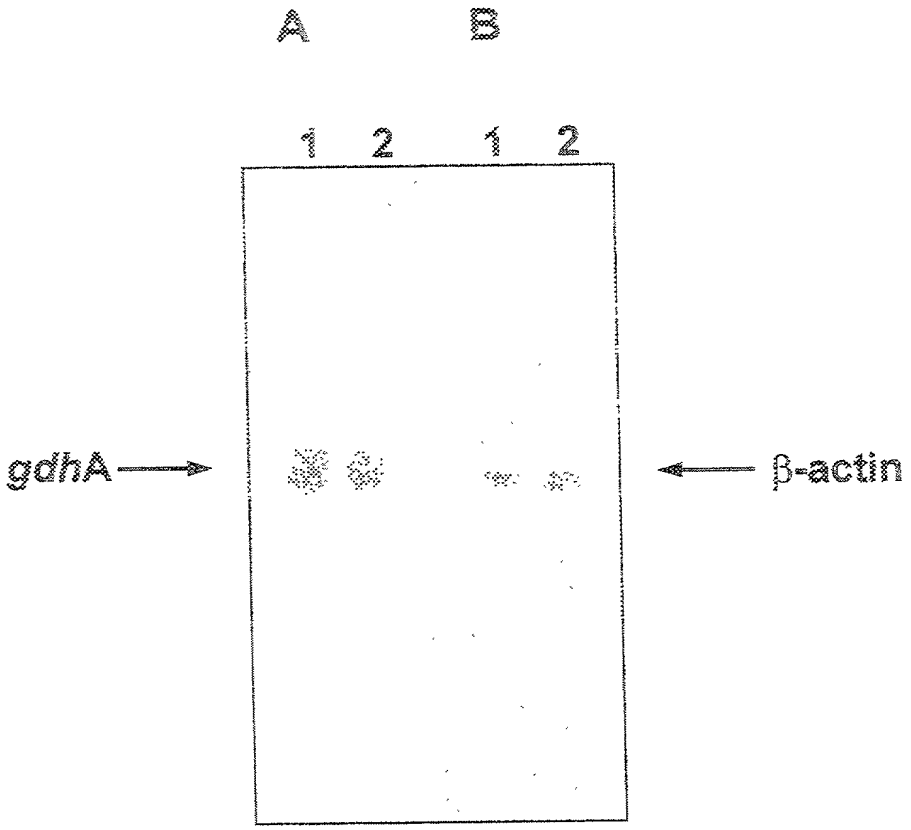
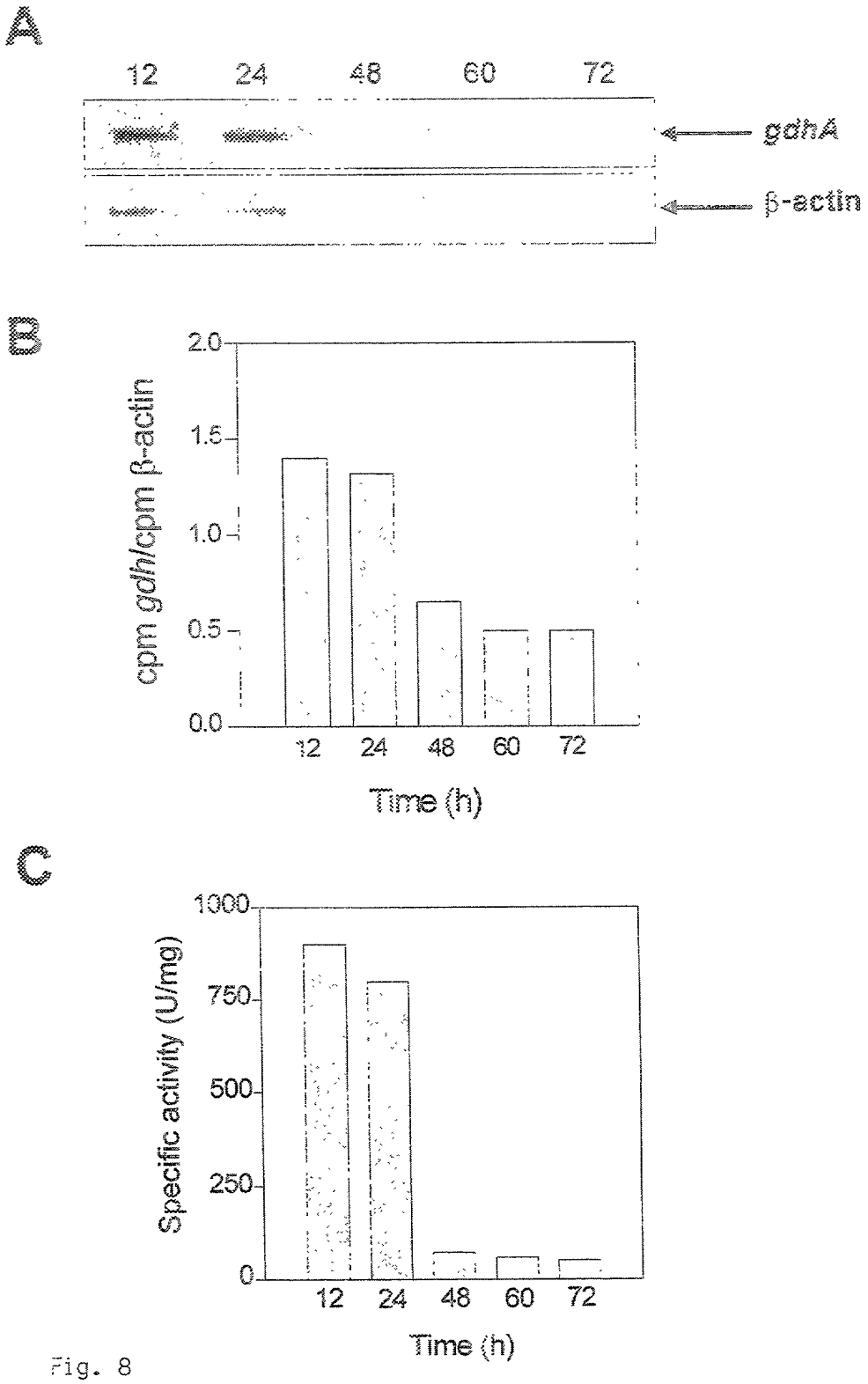


Fig. 7

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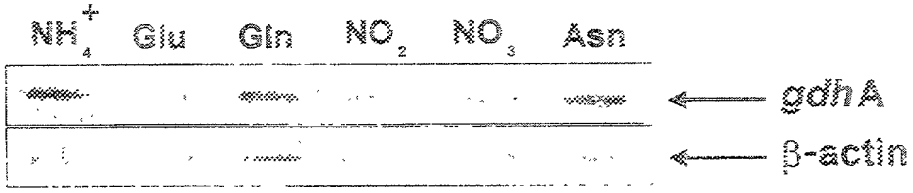
12
24
48
60
72
gdhA
β-actin
B
cpm gdh/cpm β-actin
2.0
1.5
1.0
0.5
0.0
12
24
48
60
72
Time (h)
C
Specific activity (U/mg)
1000
750
500
250
0
12
24
48
60
72
Time (h)

Fig. 8

Fig. 9

A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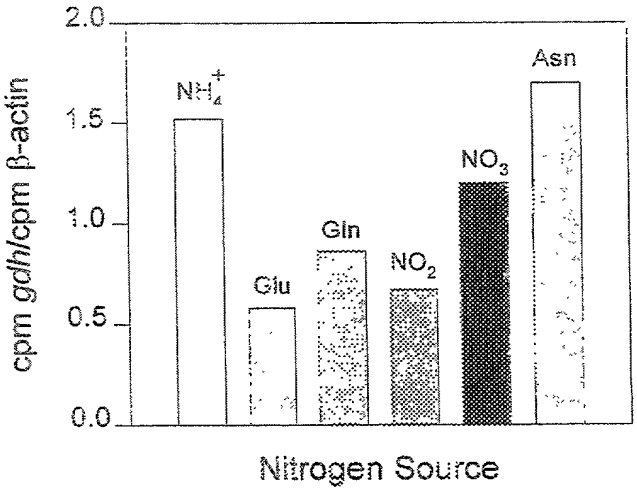
Glu
Gln
NO_2
NO_3
Asn
gdhA
β-actin

B


cpm gdh/cpm β-actin
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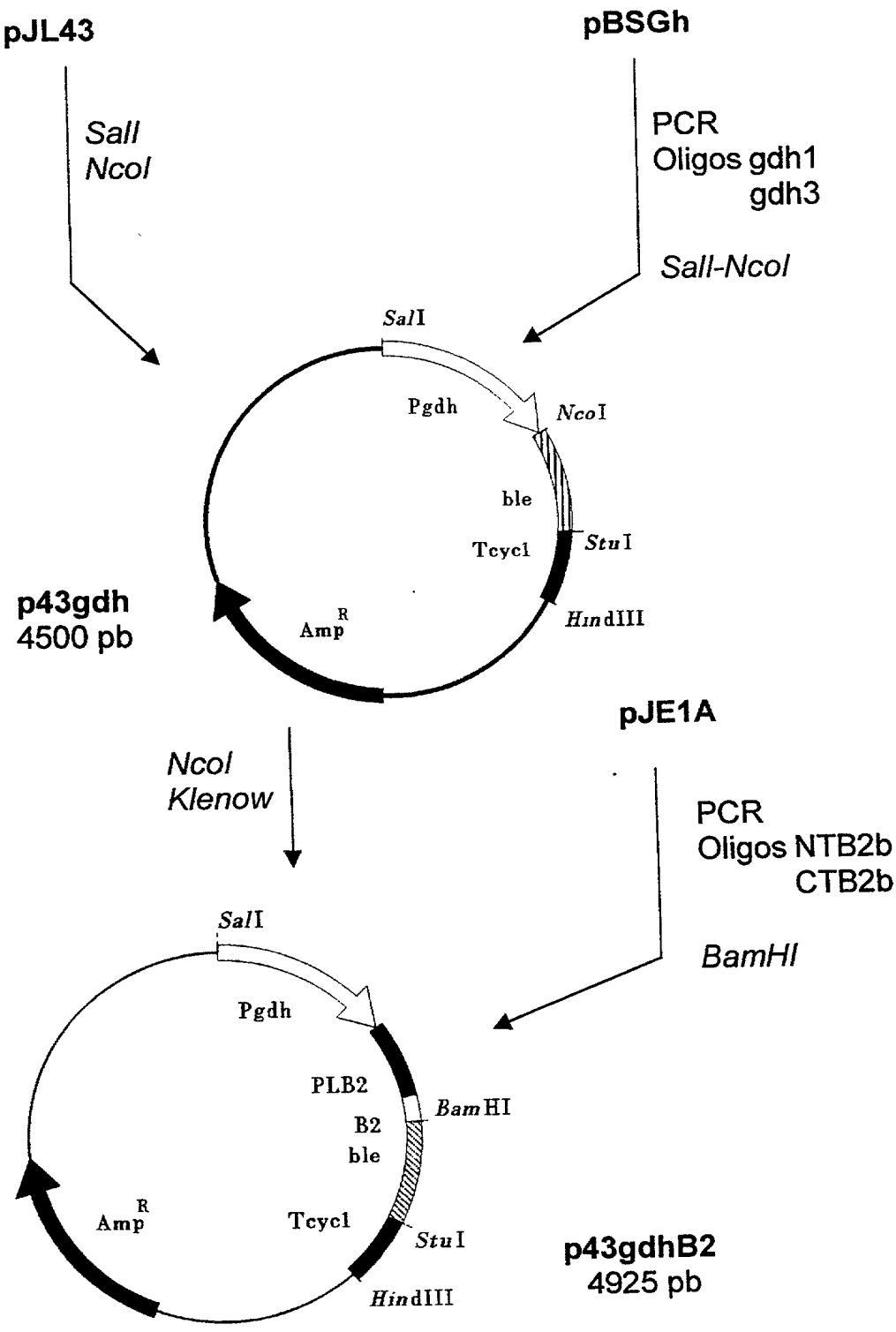
1.0
0.5
0.0
NH_4^+
Glu
Gln
NO_2
NO_3
Asn
Nitrogen Source

Fig. 10A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SalI
NcoI
PCR
Oligos gdh1
gdh3
SalI-NcoI
SalI
Pgdh
NcoI
ble
Tcycl
StuI
HindIII
Amp R
p43gdh
4500 pb
NcoI
Klenow
pJE1A
PCR
Oligos NTB2b
CTB2b
BamHI
SalI
Pgdh
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ble
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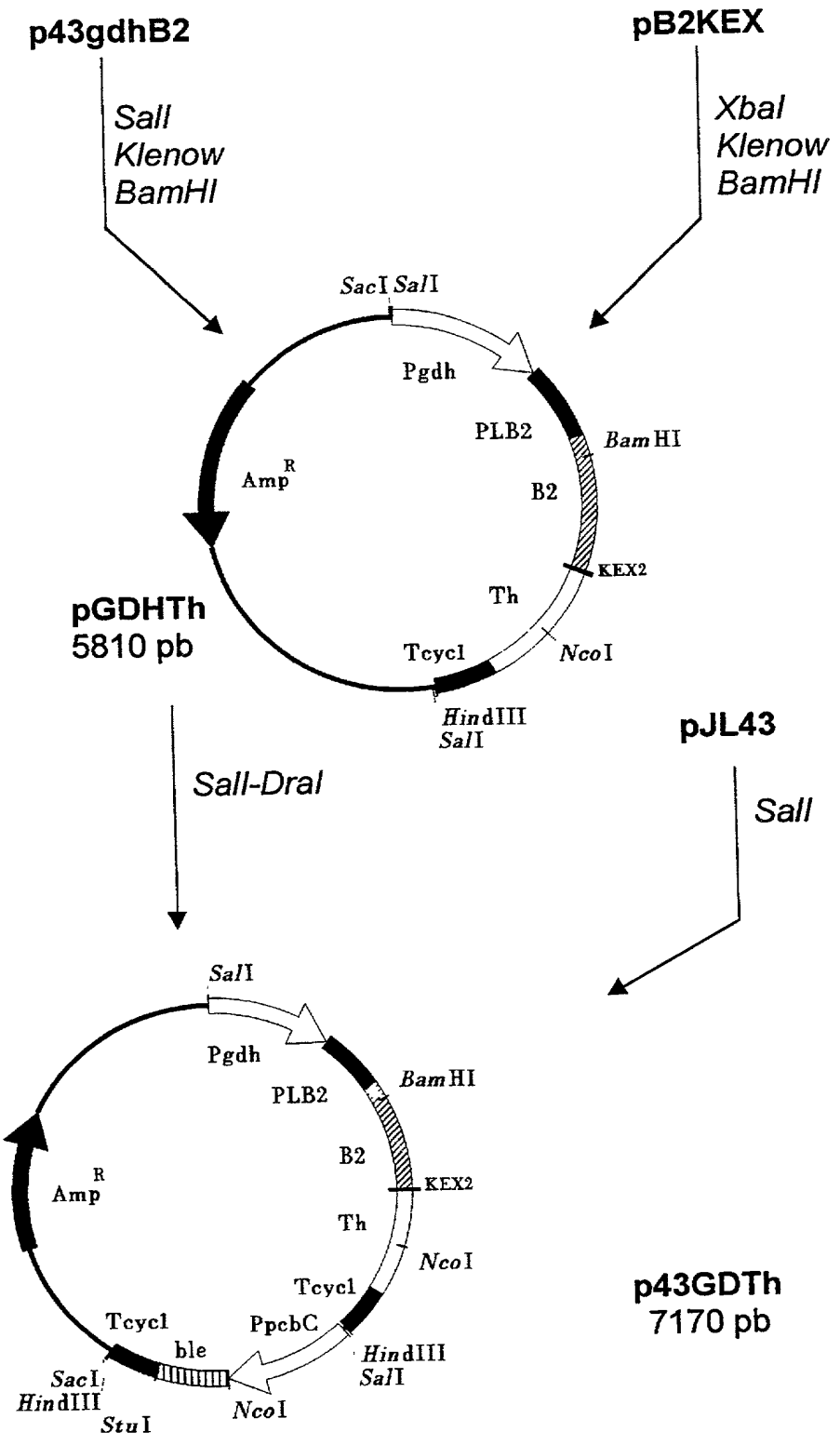
Amp R
p43gdhB2
4925 pb

Fig. 10B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XbaI
Klenow
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BamHI
B2
KEX2
Th
NcoI
Tcycl
HindIII
SalI
AmpR
pGDHTh
5810 pb
SalI-DraI
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SalI
SalI
Pgdh
PLB2
BamHI
B2
KEX2
Th
NcoI
Tcycl
PpcbC
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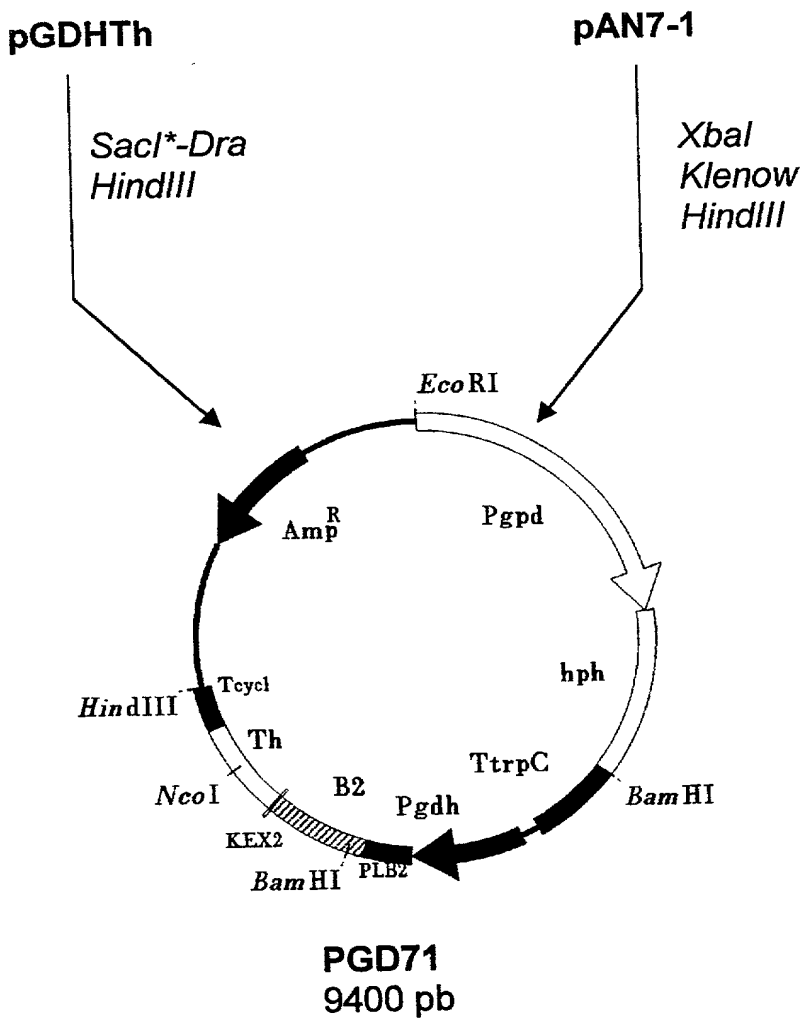
7170 pb

Fig. 10C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HindIII
XbaI
Klenow
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AmpR
Pgpd
hph
HindIII
Tcycl
Th
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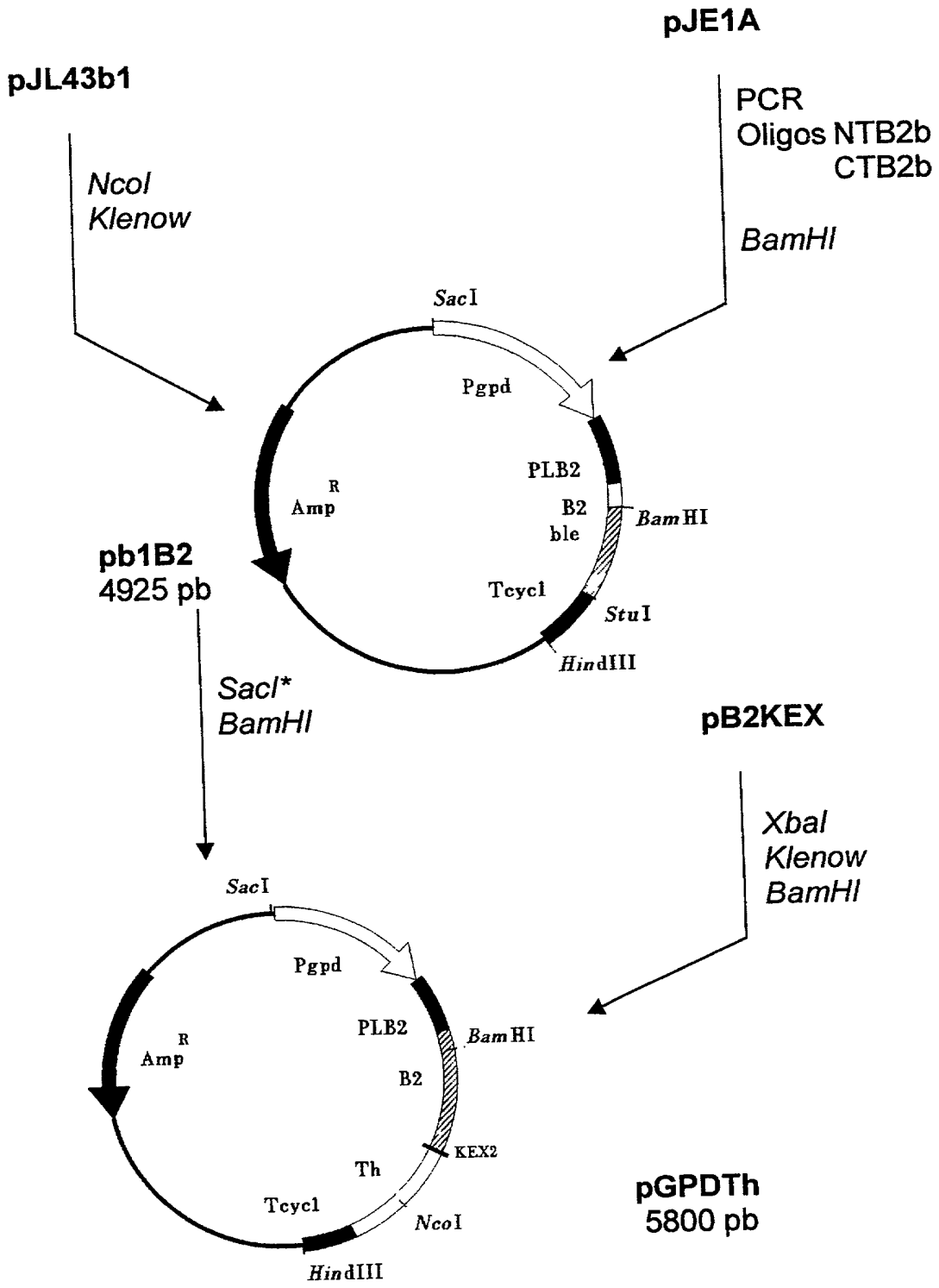
NcoI
B2
Pgdh
BamHI
KEX2
BamHI
PLB2
PGD71
9400 pb

Fig. 11A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SacI
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B2
ble
BamHI
R
Amp
Tcycl
StuI
HindIII
pb1B2
4925 pb
SacI*
BamHI
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XbaI
Klenow
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SacI
Pgpd
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Fig. 11B

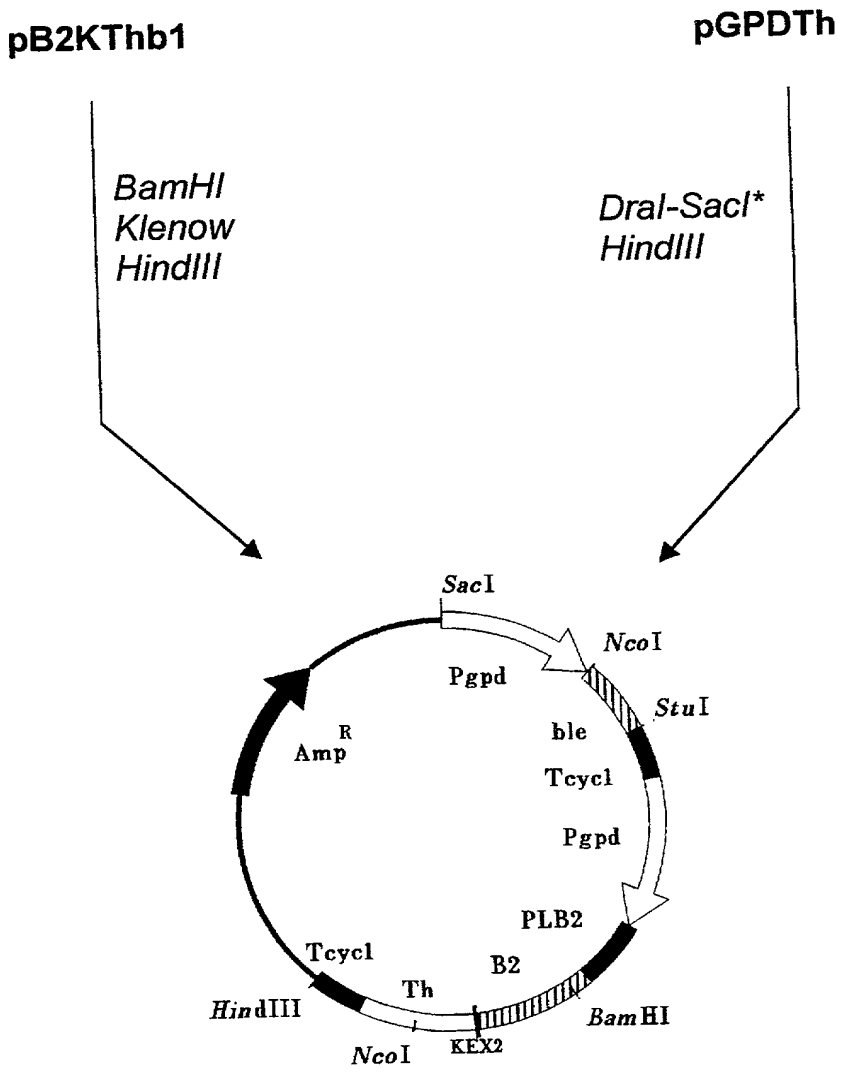

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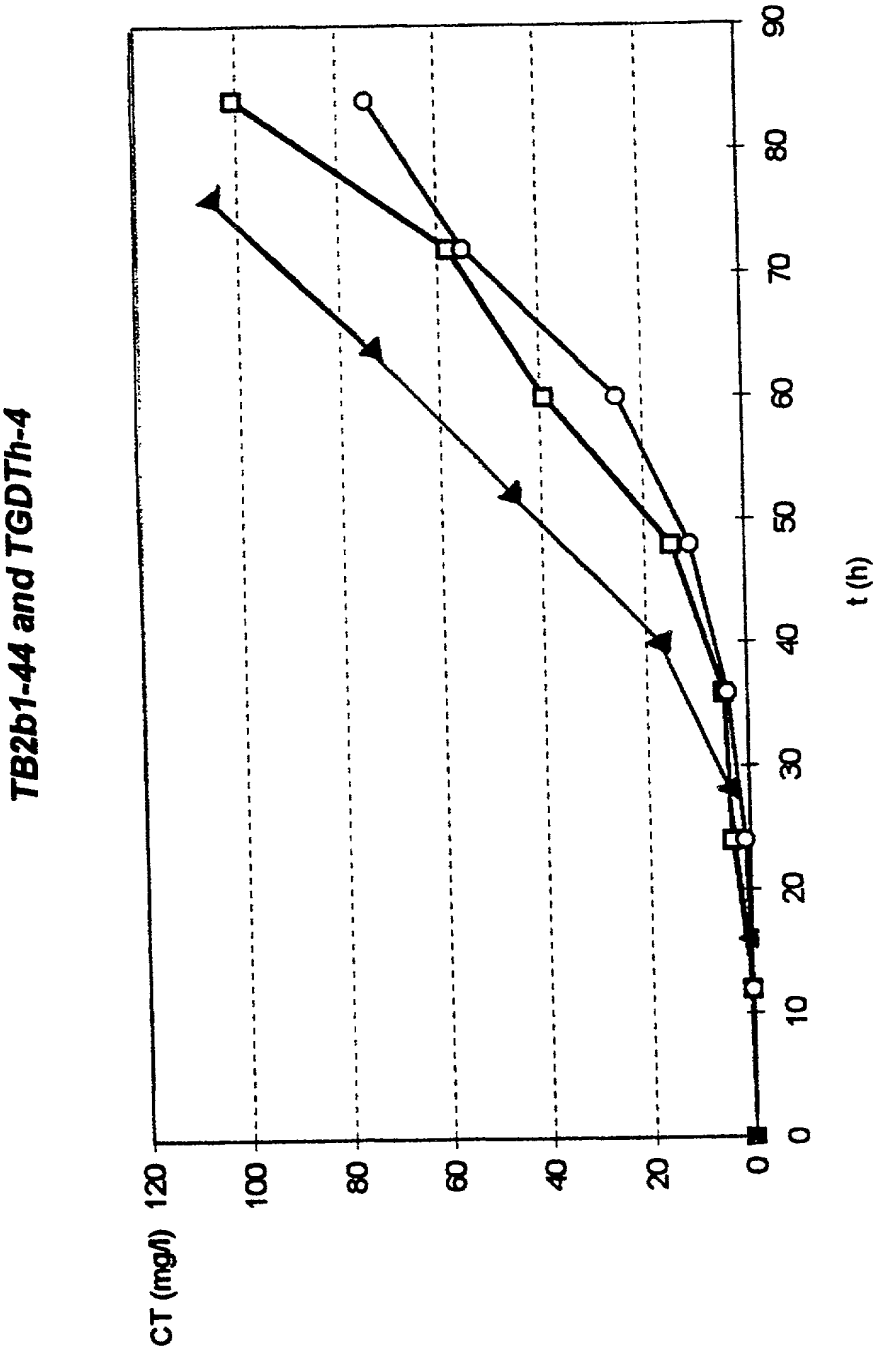
Fig. 12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CT (mg/l)
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100
80
60
40
20
0
t (h)
0
10
20
30
40
50
60
70
80
90

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 031309-003

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name;
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROMOTER AND CONSTRUCTIONS FOR EXPRESSION OF RECOMBINANT PROTEINS IN FILAMENTOUS FUNGI

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application
Number _____
on _____
and was amended
on _____ (if applicable).

☒ was filed as PCT international application
Number PCT/EP99/02243
on 1 April 1999
and was amended
on 5 June 2000 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(e) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119:

COUNTRY (If PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. §119
Spain (ES)	98 00699	2 April 1998	X Yes _ No
			_ Yes _ No
			_ Yes _ No
			_ Yes _ No

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

_____ (Application Number) _____ (Filing Date)

_____ (Application Number) _____ (Filing Date)

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONT'D) (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 031309-003

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Office all information known to me to be material to the patentability as defined in Title 37, Code of Federal Regulations §1.56, which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120:

U.S. APPLICATIONS		STATUS (check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. APPLICATION NUMBERS ASSIGNED (if any)			
PCT/EP99/02243	1 April 1999	-		✔	

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(5/00)

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONT'D) (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 031309-003

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONT'D) (Includes Reference to Provisional and PCT International Applications)		Attorney's Docket No.

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONT'D) (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 031309-003

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (CONT'D) (Includes Reference to Provisional and PCT International Applications)	Attorney's Docket No. 031309-003

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